

**Investigation the Effects of Cigarette Smoke on Immunoglobulin
Levels in Serum and Saliva Samples of Smoker and Non-Smoker
Subjects Using Antibody-Microarray Technology**

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Abstract

Cigarette smoke (CS) has many damaging effects on the body, and the chronic inhalation of cigarette smoke can change immunological functions through impact on both innate and adaptive immunity. The incidences of many diseases are affected by the adverse effects of cigarette smoke on the immune system, and the induction of an inflammatory response, which affects several tissues and organs. On this basis, a comparison of smokers' and non-smokers' immunoglobulin levels could provide valuable insights into the mechanisms of smoking related diseases. Although the effects of cigarette smoking on humoral and cellular immunity have been investigated previously, the results have varied between the studies, and therefore more research is still required.

The aim of this study was to determine whether the levels of immunoglobulin (Ig) isotypes are different in the serum and saliva of non-smoking individuals compared to smoking individuals. An examination of serum and saliva would provide information on the effects of cigarette smoke systemically and in the oral mucosa, respectively. The effects of cigarette smoke extract on B-cell secretions were also examined to establish whether cigarette smoke components can have direct effects on immunoglobulin production by B cells. In order to determine Ig isotype levels, antibody microarray techniques were established and calibrated for determining the sample concentrations of IgM, IgG, IgA and IgD. The results showed that smoking has different effects on systemic and salivary immunoglobulin levels. In the serum, smokers had decreased levels of IgG and IgD, but increased IgM and IgA levels compared to non-smokers. However, in the saliva smokers had decreased levels of IgG, IgD, and IgM, whereas there were increased levels of IgA in smokers' saliva. As CS has been found to influence the serum and salivary levels of Ig isotypes *ex-vivo*, the mechanisms underlying these effects were investigated *in vitro* to determine whether the changes were as a result of a direct effect of the CS on B-cells. This study has shown that CS had deleterious effects on the production and the levels, of Ig isotypes. These results support the concept that CS is related to diseases, and more research is necessary in this field.

Declaration

I hereby declare that this thesis is my own work and it based on research that was undertaken in Immunology at the School of Life Sciences, University of Nottingham, and has not been previously submitted for any other degrees.

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Table of contents

Abstract	ii
Declaration	iii
Acknowledgements	iv
Table of contents	vi
List of figures.....	xiii
List of tables	xvii
Abbreviations	xviii
Conferences and meetings attended.....	xxi
 1. General Introduction.....	 1
1.1. Innate immunity	1
1.2. Adaptive immunity	2
1.2.1. Cell-Mediated immune response	2
1.2.1.1. T-lymphocytes	2
1.2.1.1.1. T-helper cells (Th)	4
1.2.1.1.2. T-cytotoxic cells (Tc).....	4
1.2.1.1.2.1. Perforin and granzyme mediated apoptosis	6
1.2.1.1.2.2. FAS-mediated apoptosis	6
1.2.1.1.3. T-regulatory (Treg) cells.....	7
1.2.1.2. Macrophages.....	8
1.2.1.3. NK cells	9
1.2.2. Humoral-immune response (B-cells)	9
1.2.2.1. Sub-populations of B cells	11
1.2.2.1.1. B-1 cells	11
1.2.2.1.2. B-2 cells	11
1.2.2.1.3. MZ B cells	11
1.2.2.1.4. Naïve B cells	11

1.2.2.1.5. FO B cells	11
1.2.2.1.6. Memory B lymphocytes.....	11
1.2.2.1.7. Plasma cells.....	12
1.2.2.2. Stages of B cell development and differentiation	12
1.2.2.2.1. Phase I: Antigen independent differentiation.....	12
1.2.2.2.2. Phase II: Antigen dependent differentiation	16
1.2.2.3. B cell transcription factors	17
1.2.2.4. Surface markers	18
1.2.2.5. Activation of B cells	18
1.2.2.5.1. T-cell independent activation.....	18
1.2.2.5.2. T cell dependent activation	18
1.2.2.6. Role of cytokines in B cell maturation and activation	19
1.2.2.7. B cell class switching.....	20
1.2.2.8. Antigen-antibody binding	20
1.2.2.9. Immunoglobulins (Ig)	22
1.2.2.9.1. Immunoglobulin G (IgG).....	23
1.2.2.9.2. Immunoglobulin M (IgM).....	24
1.2.2.9.3. Immunoglobulin A (IgA).....	24
1.2.2.9.4. Immunoglobulin D (IgD).....	26
1.2.2.9.5. Immunoglobulin E (IgE).....	27
1.2.2.10. Function of antibodies	28
1.2.2.10.1. Agglutination	29
1.2.2.10.2. Opsonization	29
1.2.2.10.3. Antibody-dependent cellular cytotoxicity (ADCC)	29
1.2.2.10.4. Complement mediated lysis and inflammation.....	29
1.2.2.10.5. Neutralization.....	29
1.3. Cigarette smoke and the immune system.....	30
1.3.1. Mechanisms of how cigarette smoke extracts exert inflammatory and suppressive effects on immune cells	31

1.3.2. Mechanism of how CS alters mucosal immunity	32
1.3.3. Cigarette smoke and innate immunity	34
1.3.4. CS and adaptive immunity.....	35
1.3.5. Effects of CS on Immunoglobulins.....	36
1.4. Protein microarray overview ^[1] _{SEP}	38
1.4.1. Microarray slide surfaces and immobilisation	39
1.4.2. Spot size and morphology.....	40
1.4.3. Spot background	41
1.4.4. Signal detection and generation.....	41
1.4.5. Assay validation.....	43
1.4.5.1. Parameters of assay validation.....	45
1.4.5.1.1. Specificity	45
1.4.5.1.2. Intra assay variation	45
1.4.5.1.3. Inter assay variation	45
1.4.5.1.4. Limit of detection.....	45
1.5. Aims.....	46
2. Optimisation of the ELISA Technique	47
2.1. Introduction.....	47
2.2. Materials and methods.....	47
2.2.1. Materials	47
2.2.2. Developing the ELISA technique	47
2.2.2.1. Choosing a suitable blocking buffer and concentration for the different immunoglobulin isotypes to generate standard curves	47
2.2.2.2. Cross reactivity experiments for studying the specificity of the in-house ELISA	50
2.2.2.2.1. Cross-reactivity experiments for determining IgG specificity.....	51
2.2.2.2.2. Cross-reactivity experiments for determining IgM specificity	51

2.2.2.2.3. Cross-reactivity experiments for studying the specificity of the ELISA quantitation set for detecting IgM, IgG, IgA, and IgD	51
2.3. Results.....	51
2.3.1. Choosing a suitable blocking buffer and concentration for detecting different Ig isotypes to generate standard curves.....	51
2.3.2. ELISA cross-reactivity results	53
2.3.2.1. Results of studying the specificity of the in-house ELISA with Igs	53
2.3.2.2. Specificity of the ELISA Quantitation Set in detecting IgM, IgG, IgA, and IgD	54
2.4. Discussion.....	56
2.5. Conclusions.....	56
3. Optimisation and Validation of Antibody Microarray	57
3.1. Introduction.....	57
3.2. Materials and methods	59
3.2.1. Materials	59
3.2.2. Printing slides on the arrayer	60
3.2.3. Amplification	60
3.2.4. Slide preparation	61
3.2.5 Scanning of slides	62
3.2.6 Slide analysis	63
3.3. Developing the antibody microarray	65
3.3.1. Generating an IgG standard curve	65
3.3.1.1 Determining the best printing buffer.....	65
3.3.1.2 Determining the optimal blocking buffer	65
3.3.1.3 Using a biotinylated secondary antibody	65
3.3.1.4 Using an amplification reagent and an HRP-conjugated goat anti-human IgG-Fc as a secondary antibody at different concentrations.....	66

3.3.2. Generating IgM and IgA standard curves	66
3.4. Antibody microarray validation test	66
3.4.1. Cross-reactivity test	66
3.4.1.1. Examination of the cross-reactivity for IgG with IgM and IgA by using biotinylated anti-IgG (H & L) as detection antibody	67
3.4.1.2. Examination of IgG cross-reactivity with IgM and IgA using an HRP-conjugated, goat anti-human IgG-Fc as the detection antibody	67
3.4.1.3. Examination of the cross-reactivity for IgM with IgG and IgA, also IgA with IgG and IgM.....	68
3.4.2. Precision tests	68
3.4.2.1. Intra- and inter-assay variability	68
3.5. Results.....	68
3.5.1. Developing the antibody-microarray	68
3.5.1.1. Determining the best printing buffer and blocking buffer	68
3.5.1.2. Determining the best capture-antibody concentration for printing onto slides and using a biotinylated secondary antibody.....	68
3.5.1.3. Using an amplification reagent and HRP-conjugated, goat anti-human IgG-Fc as a secondary antibody at different concentrations	71
3.5.1.4. IgM and IgA standard curves.....	71
3.5.2. Antibody microarray-validation results	74
3.5.2.1. Cross-reactivity results.....	74
3.5.2.1.1. Examination of anti-IgG cross-reactivity with IgM and IgA, using biotinylated anti-IgG (H & L) as a detection antibody	74
3.5.2.1.2. Examination of the cross-reactivity for anti-IgG with IgM and IgA when using HRP-conjugated goat anti-human IgG-Fc as a detection antibody.....	74
3.5.2.1.3. Examination of the cross-reactivity for anti-IgM with IgG and IgA, as well as that for anti-IgA with IgG and IgM	76

3.5.2.2. Precision test results.....	80
3.5.2.2.1. Intra- and inter-assay variability results.....	80
3.5.2.2.2. Limit of detection (LOD).....	81
3.6. Discussion.....	83
3.7. Conclusions.....	84
4. Comparing Antibody-Microarray Data from Smokers and Non-Smokers.....	85
4.1. Introduction.....	85
4.2. Materials and methods	85
4.2.1. Procedure for serum and saliva collection	85
4.2.1.1. Collection of demographic data on the subjects	85
4.2.1.2. Serum collection	86
4.2.1.3. Unstimulated saliva collection.....	86
4.2.2. Assaying serum and saliva samples	86
4.2.3. Applying serum and saliva samples.....	86
4.3. Results.....	89
4.3.1. Serum and saliva dilution-series results.....	89
4.3.2. Serum sample results	89
4.3.3. Saliva samples results	89
4.3.4. Studying correlations between serum immunoglobulins	93
4.3.5. Studying correlations between saliva immunoglobulins	93
4.3.6. Studying correlations between serum and saliva immunoglobulins	93
4.3.7. Correlation between immunoglobulin levels and smoking pack-years	93
4.4. Discussion.....	102
4.5. Conclusions.....	109
5. Studying the Effects of CSE/Nicotine on B-cell Secretions in vitro.....	110
5.1. Introduction.....	110

5.2. Materials and methods	110
5.2.1. Materials	110
5.2.2. Methods	111
5.2.2.1. Isolation of PBMCs	111
5.2.2.2 Cell counting of viable cells	112
5.2.2.3. Magnetic separation of B cells.....	112
5.2.2.4. Treatment of cell cultures with CSE or nicotine.....	113
5.2.2.5. Preparation of CSE	113
5.2.2.6. Determination of Ig levels in cell-free supernatants	115
5.2.2.7. Statistical analysis.....	115
5.3. Results.....	116
5.4. Discussion.....	118
5.5. Conclusions.....	118
6. General Discussion.....	119
6.1. Conclusions and future work	123
References.....	126
Appendix.....	149

List of figures

Figure 1.1: Stem cell development.	3
Figure 1.2: Overview of the adaptive immune response after antigen recognition.	5
Figure 1.3: Effector cytotoxic T cells killing of target cells by two strategies.	8
Figure 1.4: Antibody structure.....	10
Figure 1.5: B cell differentiation in the bone marrow (BM) and periphery occurs in multiple steps.	14
Figure 1.6: Membrane Ig in pre-B cells.....	15
Figure 1.7: Stages of B cell differentiation.....	15
Figure 1. 8: B-cell development.....	16
Figure 1.9:The interaction between Ag and B cell surface antigen receptor.	21
Figure 1.10: Schematic illustration of the immunoglobulins H and L chain modules.	23
Figure 1.11: CS is a combination of thousands of chemical compounds, produced from the burning of tobacco.	31
Figure 1.12: CS regulates inflammation and stimulates chronic inflammation in the airways by various mechanisms.....	34
Figure 1.13: Picture of the BioRobotics MicroGrid II Arrayer.	40
Figure 1.14: Diagram showing tyramide signal amplification (TSA).	43
Figure 2.1: Graphical illustration of the principle of the ELISA technique.....	50
Figure 2.2: In-house developed ELISA, comparison of the results obtained using three different blocking buffers.	53
Figure 2.3: Cross-reactivity experiments for the in-house ELISA.	54
Figure 2.4: Investigation of cross-reactivity between anti-Ig capture and detection antibodies of different Ig class specificities with Ig standards of different classes.	55
Figure 3.1: Principle of the sandwich antibody microarray	62

Figure 3.2: Diagram of the microarray assay protocol for quantification of total Ig antibodies.....	64
Figure 3.3: Schematic diagram shows the steps for generating the numerical numbers and curves.....	64
Figure 3.4: Determining the specificity of capture and detection antibodies.	67
Figure 3.5: Determining blocking buffer suitability.	69
Figure 3.6: Determining the capture antibody concentration.	70
Figure 3.7: Different dilution of IgG used for standard curves.	70
Figure 3.8: Examining different concentrations of an HRP-conjugated, goat anti-human IgG-Fc secondary antibody to improve the IgG standard curve.	72
Figure 3.9: IgG standard curves generated on three different days. A secondary antibody dilution of 1:800,000 was chosen for the IgG standard curve.	72
Figure 3.10: Examination of the different concentrations of the HRP-conjugated goat anti-human IgM and anti-IgA.	73
Figure 3.11: Cross-reactivity experiments.....	75
Figure 3.12 (a): Studying cross-reactivity between IgM, IgA and IgD standards with anti-IgG.	75
Figure 3.12 (b): Studying potential binding between IgG and different capture antibodies.....	76
Figure 3.13 (a): Studying the cross-reactivity between IgG, IgM standards and IgD with anti-IgA antibodies.	77
Figure 3.14 (a): Studying the cross-reactivity between IgG and IgA standards with anti-IgM antibodies.	78
Figure 3.13 (b): Studying potential binding between IgA and different captures antibodies.....	78

Figure 3.14 (b): Studying potential binding between IgM and different captures antibodies.....	79
Figure 3.15: Example of slides used for studying the cross-reactivity of IgG and IgA standards with the IgM capture and detection antibodies.	79
Figure 3.16: The coefficients of variation (CVs) of intra-assay results were calculated for several samples.	80
Figure 3.17: The percent coefficient of variation (CV %) for inter-assay test results was calculated for several serum samples.....	81
Figure 3.18: Determining the LLOD for IgG.	82
Figure 4.1: Determination of a suitable serum dilution.	90
Figure 4.2: Comparing the Ig concentrations in serum samples from smokers and non-smoker subjects using a Mann–Whitney test revealed significant differences.....	91
Figure 4.3: Comparing Ig concentrations in saliva samples from smokers and non-smoker subjects using a Mann–Whitney test revealed significant differences.....	92
Figure 4.4: The Spearman correlation coefficient (2-tailed) between paired Ig isotypes for smoker’s serum samples.	94
Figure 4.5: The plot of the Spearman correlation coefficient (2-tailed) between paired Ig isotypes for non- smoker’s serum samples.	95
Figure 4.6: The plot of the Spearman correlation coefficient (2-tailed) between paired Ig isotypes for smokers’ saliva samples.....	96
Figure 4.7: The plot of the Spearman correlation coefficient (2-tailed) between paired Ig isotypes for non-smokers saliva samples.....	97
Figure 4.8: The plot of the Spearman correlation coefficient (2-tailed) between Ig isotypes in saliva and serum samples for smoker’s subjects.	98
Figure 4.9: The plot of the Spearman correlation coefficient (2-tailed) between Ig isotypes in saliva and serum samples for non-smoker’s subjects.	99

Figure 4.10: Studying the relation between serum Ig levels and smoking pack-years...	100
Figure 4.11: Studying the relation between saliva Ig levels and smoking pack-years. ..	101
Figure 5.1: Preparation of CSE.....	114
Figure 5.2: Using a NanoDrop spectrophotometer to analyse CSE absorbance in a standard curve.	115
Figure 5.3: Stimulated human PBMCs were incubated for 8 days in the presence of anti-CD40, IL-4, PWM, 3 % CSE, or nicotine.	116
Figure 5.4: Stimulated human B cells were incubated for 8 days in the presence of anti-CD40, IL-4, PWM, 3 % CSE, or nicotine.	117
Figure 6.1: Graph of the levels of circulating cytokines in smokers and non-smokers...	121

List of tables

Table 1.1: Details of how the cell-mediated immune response involves T cells, macrophages and natural killer cells.....	3
Table 1.2: Summary of the classes of immunoglobulins [66].	28
Table 2.1: Reagents and materials used for the ELISA technique.	48
Table 2.2: Concentrations of specific capture antibodies used for all Ig isotypes, as well as their sources.	48
Table 3.1: List of materials used for the microarray technique.....	59
Table 4.1: Demographics of the smoker subjects (a).....	87
Table 4. 2: Demographics of the smoker subjects (b).....	88
Table 5.1: List of materials used for <i>in vitro</i> experiments.....	111

Abbreviations

AAPS	American Association of Pharmaceutical Scientists
ADCC	antibody dependent cell mediated cytotoxicity
Ag	antigen
AhR	aryl hydrocarbon receptor
AMs	alveolar macrophages
AP-1	activatory protein-1
APC	antigen presenting cells
BALF	broncho-alveolar lavage fluid
BCR	B-cell receptor
BM	bone marrow
BSA	bovine serum albumin
C	constant region
Ca ²⁺	calcium
CAD	caspases activate deoxy ribonucleases
CD	cluster of differentiation
CDRs	complementarity determining regions
COPD	chronic obstructive pulmonary disease
CRP	C-reactive protein
CS	cigarette smoke
CSE	cigarette smoke extract
CSR	class-switch recombination
CTL	cytotoxic T lymphocyte
CV	coefficients of variation
D	diversity
DNA	deoxyribonucleic acid
EDTA	Ethylene diamine tetra-acetic acid
EIA	enzyme immunoassay
ELISA	Enzyme-Linked Immuno-Sorbant Assay
Fab	fragment antigen binding
FasL	Fas ligand
Fc	fragment crystallisable
FO	follicular

GC	germinal centres
H	heavy Chain
H ₂ O ₂	hydrogen peroxide
HIV	human immunodeficiency virus
HRP	horseradish peroxidase
IFN- γ	interferon-gamma
Ig	immunoglobulin
IL	interleukin
IL-7R	IL-7 receptor
IRF8	interferon regulatory factor 8
ITAM	immunoreceptor tyrosine based activation motif
J	joining
L	light Chain
LDL	Low density lipoprotein
LLOD	lower limit of detection
LOD	limit of detection
LPS	lipopolysaccharide
MALTs	mucosa-associated lymphoid tissues
MAPK	mitogen-activated protein kinases
MHC	major histocompatibility complex molecules
mIg	membrane Ig
MZ	marginal zone
NaCl	sodium chloride
NF- κ B	nuclear factor kappa-B
NH ₂	amino
NK	natural Killer
O ⁻	superoxide
OH ⁻	hydroxyl
PAX	paired box gene
PBMCs	peripheral blood mononuclear cells
PBS	phosphate buffered saline
pCTLs	precursor cytotoxic CD8 ⁺ T lymphocytes
pre-BCR	pre-B-cell receptors

pro-B cell	progenitor B cells
ROO [•]	peroxyl radicals
ROS	reactive oxygen species
SD	standard deviation
SHM	somatic hypermutation
sIg	surface immunoglobulin
sIgA	secretory IgA/ salivary IgA
STAT	signal transducer and activator of transcription
T reg	T regulatory
T1	transitional 1
T2	transitional 2
Tc-cell	T-cytotoxic cell
TCF3	transcription factor E2-alpha
TCR	T-cell-receptor
TdT	terminal deoxynucleotidyl transferase
TGF- β	transforming growth factor-beta
Th-cell	T-helper
TLR	Toll-like receptor
TMB	tetra methyl benzidine
TNF- α	Tumour necrosis factor alpha
US FDA	U. S. Food and Drug Administration
UV	Ultra violet
V	Variable
VCAM-1	Vascular cell adhesion molecule-1
VLA-4	adhesion molecule
λ 5	lambda 5

Conferences and meetings attended

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- British Society for Immunology Congress (BSI), Liverpool, UK; 2013.
- British Society for Immunology Congress (BSI), Brighton, UK; 2014.
- Immunity Group Meeting (BSI), Oxford, UK; 2014.
- Inflammation & disease conference (BSI), Manchester; UK; 2014.
- The 7th Saudi Students Conference, Edinburgh, UK; 2014.
- The Infection & Immunity Group Meeting (BSI), Leeds, UK; 2014.
- The 4th European Congress of Immunology (ECI), Vienna, Austria; 2015.
- The 9th Saudi Students Conference, London, UK, 2016.

1. General Introduction

Smoking is considered as one of the crucial causes of morbidity and mortality worldwide, and there are numerous diseases which are correlated with smoking habits such as cardiovascular disease, and lung disease [1-3]. It is known that cigarette smoke has many toxic components such as nicotine, and these components have immunomodulatory properties [4, 5]. Studies have shown that nicotine for example can cause inhibition of the innate and the adaptive immune system; immunological irregularities and suppression of the immune system which could play a role in the incidence of certain conditions and diseases [6]. Also, cigarette smoke has been shown to decrease the function of immunocompetent cells in experimental animals both *in vitro* and *in vivo*, and it has been shown that smokers have reduced quantities of serum immunoglobulins and an altered antibody response to the influenza virus after natural infection and vaccination [5, 7]. Although, smoking has been shown to change the immunological response to antigens; for example in the pathogenesis of allergic alveolitis and respiratory infection, more research is required to fully evaluate the biological effects of cigarette smoke, especially nicotine, on the immune system [5, 8].

1.1. Innate immunity

Innate immunity refers to the non-specific defence mechanisms that include physical barriers such as the skin, chemicals, and immune cells that attack foreign materials in the body. It works immediately or within hours of invaders attacking the body. The major roles of innate immunity involve it acting as a physical and chemical barrier to infectious agents; the dead cellular layer of the skin acts as a barrier to infection, and the mucous membranes secrete enzymes that can destroy bacteria, produce mucus that entraps microbes and there are cells expressing cilia that filter pathogens. The innate immune response causes recruitment of immune cells to places of infection through release of chemical factors called cytokines, stimulation of the complement cascade to help eliminate bacteria, and assisting in the identification and removal of foreign substances that exist in organs, tissues, the blood and lymph. Additionally, it can activate the adaptive immune system through the presentation of antigens by antigen presenting cells (APC) and phagocytes such

as neutrophils which phagocytize bacteria; eosinophils which secrete enzymes to kill parasitic worms, and macrophages which are the main tissue phagocytic cells against many pathogens [9-11].

1.2. Adaptive immunity

The B and T lymphocytes are generated from active lymphoid stem cells; the main site of B cell production is the liver in the foetus and the bone marrow in adults, whereas T-cells are produced from bone-marrow pre-T cells that migrate to the thymus for development and maturation (Figure 1.1). The adaptive immunity is activated when the innate immunity alone fails in eliminating the pathogen. Antigen presenting cells, such as dendritic cells, B cells and macrophages, engulf the pathogen, process it and present it in association with major histocompatibility complex molecules (MHC), which interact with and activate the antigen-specific T lymphocytes triggering the adaptive immune response. Adaptive immunity is more effective, specific and has a “memory” that creates a more efficient response in case re-infection happens in the future; its response can be divided into the cell-mediated immune response and the antibody-mediated (humoral) immune response [12, 13].

1.2.1. Cell-Mediated immune response

Cell-mediated immunity includes the activity of T cells, macrophages and natural killer cells, which all have specific roles (Table 1.1).

1.2.1.1. T-lymphocytes

In the thymus, T cells differentiate into two different forms: T-helper (Th-cell), which express cell-surface cluster of differentiation CD4 proteins, and T-cytotoxic cell (Tc-cell) which have CD8 cell-surface proteins. The main function of CD4⁺ and CD8⁺ is to regulate which molecules can interact with the T-cell. T-cells recognize the pathogens through APCs; APCs that have MHC class II can only bind with CD4⁺ on the surface of Th-cells, while APCs with MHC class I can only bind with CD8⁺ on the surface of Tc-cells. Therefore, APCs are highly specific and depend on the type of MHC presented [13].

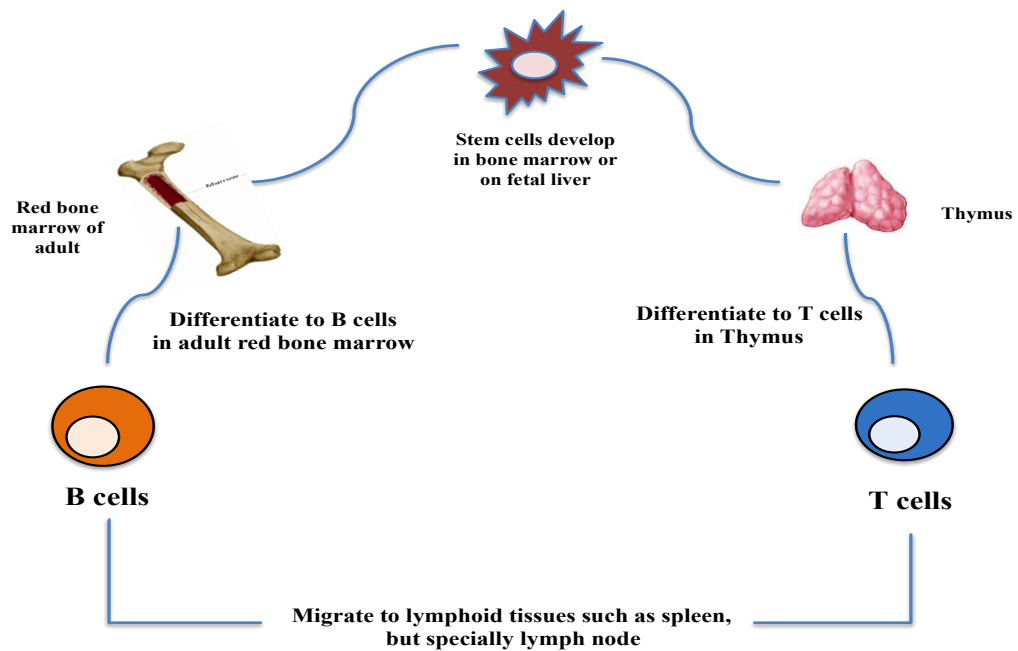


Figure 1.1: Stem cell development.

Stem cells develop in bone marrow or in foetal liver; then migrate to bone marrow and thymus. Bone marrow is the major site for B cells production, and thymus is the major site for T cell production.

Table 1.1: Details of how the cell-mediated immune response involves T cells, macrophages and natural killer cells.

Cell	Function
T helper (Th1)	Activates cells related to cell-mediated immunity – macrophages, Tc cells and natural killer cells
T helper (Th2)	Stimulates production of eosinophils, IgM and IgE
Cytotoxic T lymphocyte (CTL)	Destroys target cells
T regulatory (T reg) cell	Regulates immune response and helps maintain tolerance
Activated Macrophage	Enhances phagocytic activity, attacks cancer cells
Natural Killer (NK) cell	Attacks and destroys target cells; participates in antibody-dependent cell-mediated cytotoxic

1.2.1.1.1. T-helper cells (Th)

The activation of Th cells occurs through three steps: the direct interaction between the Th cells and APCs stimulate activation and differentiation of Th, and then secretion of cytokines. These results in activation of B cells to plasma cells or T cells to effector cells dependent on the types of cytokines involved. To start differentiation of Th cells, APCs secrete IL-1, which stimulates the Th cells to secrete interleukin 2 (IL-2) that has an autocrine (self-stimulating) function and causes further proliferation and maturation of Th cells to functional helper cells. At this stage, Th cells can differentiate into different subsets Th1, Th2 or Th17 cells, which differ in the types of cytokines that they produce. Th1 cells can help in developing cell-mediated immunity, Th2 cells work toward developing humoral immunity, and Th17 cells help in the stimulation of macrophages [12]. Additionally, the immune response may change between activating humoral or cell-mediated immunity because Th1 cells can inhibit the activity of Th2 cells and vice versa. For example, antigens resulting from viral, bacterial pathogens or cancer cells appear to produce a larger amount of Th1 cells compared to Th2 cells, whereas antigens resulting from multicellular parasites and allergens may lead to an increased Th2 cell production. However, several antigens such as the tetanus vaccine can induce humoral and cell-mediated responses simultaneously [14]. As the Th cells are essential for the production of the humoral and cellular immune responses, the destruction of these cells by some viruses, such as the human immunodeficiency virus (HIV), leads to life-threatening diseases [12, 15, 16].

1.2.1.1.2. T-cytotoxic cells (Tc)

Cytotoxic T cells (CTL) are activated when the T-cell-receptor (TCR) of a CD8⁺ Tc cell recognizes a cell which was infected with a virus or any other intracellular pathogen that is presented through MHC class I molecules. There are two ways of activation, the first occurs through CD4⁺ Th1 cells which recognize the virus through MHC class II and secrete IL2 that binds to the Tc cells and activates them to attack the pathogen infected cells; the other activation occurs when dendritic cells present the antigen by MHC class I and that leads to recognition of the pathogen by CD8⁺ Tc-cells (Figure 1.2). Once activated the number of Tc cells increases and they travel throughout the body to search for antigen-positive cells [17].

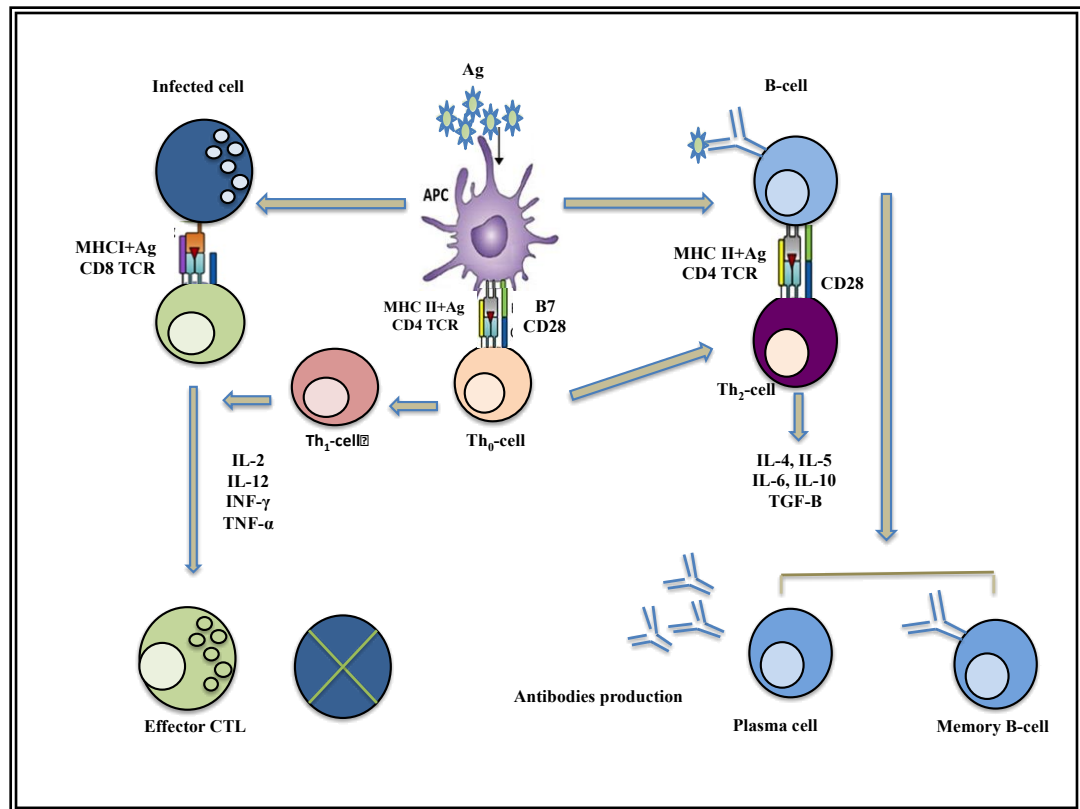


Figure 1.2: Overview of the adaptive immune response after antigen recognition.

Infected cells present antigen peptides by MHC I molecules on the plasma membrane. The peptide-MHC I complex is recognized by precursor cytotoxic CD8⁺ T lymphocytes (pCTLs). pCTLs are activated by two pathways: in the first pathway Th1 cells, induced by antigen presenting cells, produce IL-2, interferon-gamma (IFN-γ), and TNF-α. In the second pathway, dendritic cells present B7-CD28. This results in activation and differentiation of the precursor CTLs into memory or effector CTLs. Effector CTLs can directly kill infected cells by the production of perforins and granzymes. Also, activated Th2 cells stimulated by antigen presenting cells produce B cell stimulating cytokines (including IL-4, IL-5, IL-6, IL-10, TGF-β) that activate naive B cells. This causes B cell differentiation into memory B cells and plasma cells that generate IgG, IgA, IgE antibodies that block more virus infection. Ab: antibody, Ag: antigen, APC: Antigen Presenting Cell, DC: Dendritic Cell IL: Interleukin, TCR: T Cell Receptor, Th: CD4⁺ T helper cells.

The cytotoxicity of CTL and the killing of infected cells occurs in three steps – formation of the CTL-target cell conjugate, entry of granzymes into the target cell via a perforin channel, and finally apoptosis of the target cell [12]. To form a conjugate the TCR-CD3 complex binds to the antigen which was presented by MHC class I and it forms a complex with MHC class I; other accessory proteins are needed to stabilize this interaction, therefore the CD8⁺ molecule and Fas ligand on the Tc cell interact with their respective receptors on the target cell. Then, the granules in the CTL begin moving through the cytoplasm to the conjugation zone. TcL are compressed and form an immunological synapse area between the two cells.

There are two mechanisms that have been shown to kill the target cells – perforin and granzyme-mediated or Fas-mediated apoptosis [12, 18, 19].

1.2.1.1.2.1. Perforin and granzyme mediated apoptosis

Perforin proteins are released from Tc granules by exocytosis and, in the presence of calcium ions, polymerize to form a round hole of 16 nm diameter. Perforins have a domain that can interact with cell membranes of the target cells. There is some evidence to suggest that granzymes that are also released from the granules pass through the perforin hole, but other research has suggested that granzymes are internalized by receptor mediated endocytosis, and then they are released into the cytosol by a signal delivered by the perforin. There are two important granzyme types – A and B; granzyme A generates apoptosis via a caspase independent pathway. Since it is translocated to the mitochondrial membrane cytochrome C is produced, which binds and activates intracellular receptor proteins, which results in activation of caspases. The caspases activate deoxyribonucleases (CAD) that cause DNA fragments, and each DNA fragment is surrounded by cytoplasm making apoptotic blebs [12, 18, 20] (Figure 1.3).

1.2.1.1.2.2. FAS-mediated apoptosis

CTLs express Fas ligand (FasL) that binds to target cell Fas receptors, this activates caspases-8, which in turn activates caspases-3, 6 and 7. Finally, calcium dependent apoptosis occurs [21] (Figure 1.3).

1.2.1.1.3. T-regulatory (Treg) cells

These are a group of T cells that regulate the immune response by suppression. They express CD4⁺, and bind to antigens through MHC class II molecules and their differentiation is controlled by various cytokines, primarily TGF- β (transforming growth factor-beta) and IL-2. Treg cells produce very high levels of TGF- β and IL-10, and other immunosuppressive cytokines, which generally reduce the activity of Th1 and Th2 cells by suppressing antigen identification and Th cell proliferation [12, 22].

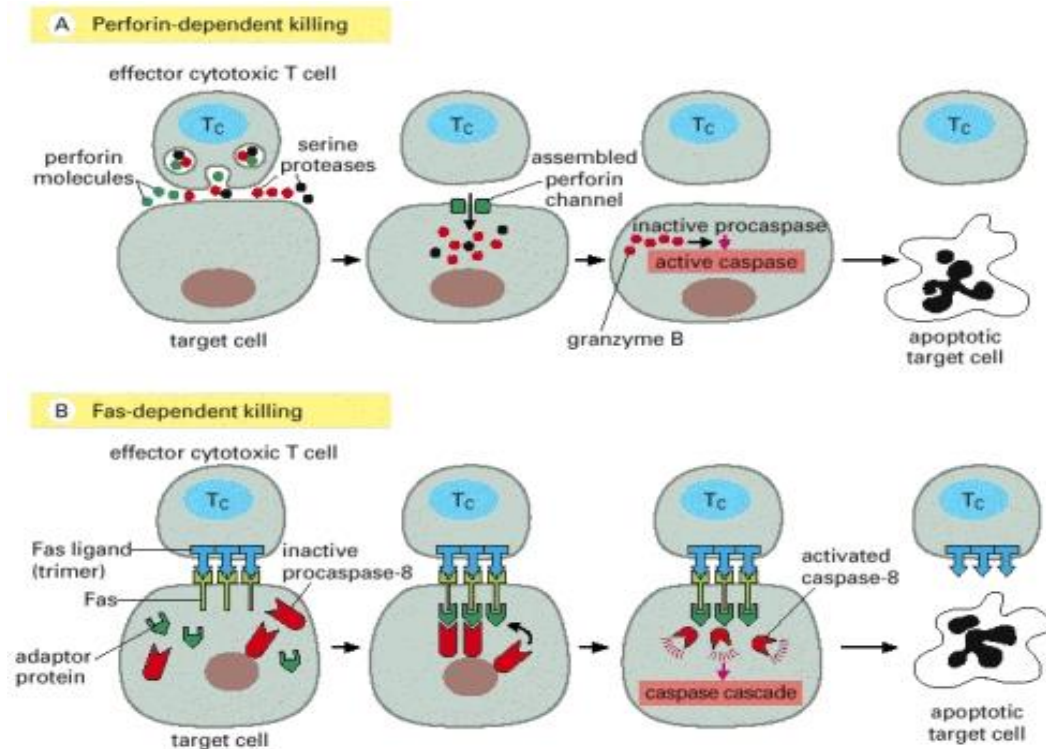


Figure 1.3: Effector cytotoxic T cells killing of target cells by two strategies.

(A) Perforin and granzyme mediated apoptosis; the cytotoxic T cell (T_C) releases perforin and proteolytic enzymes onto the surface of an infected target cell by localized exocytosis. The presence of high concentrations of Ca²⁺ in the extracellular fluid leads to the accumulation of perforin in transmembrane channels and this permits the proteolytic enzymes to arrive into the cytosol of the target cell. Then, granzyme B cleaves and triggers specific procaspases, causing activation of the proteolytic caspase cascade and apoptosis. (B) FAS-mediated apoptosis; the binding between the Fas ligand on the cytotoxic T cell surface and the Fas receptor protein on the surface of a target cell leads to activation of T_C cells. The Fas cytosolic tail includes a *death domain*, which, when activated, binds to adaptor proteins, which in turn triggers a particular procaspase (procaspase-8). Procaspase-8 molecules then split one another to yield active caspase-8 molecules that start the proteolytic caspase cascade leading to apoptosis (This figure has been reproduced from figure 24-46 of Molecular Biology of the Cell “T cell and MHC proteins chapter”, reference number [17]).

1.2.1.2. Macrophages

Macrophages stimulate apoptosis through secretion of tumour necrosis factor (TNF). If the target cell is too large to be phagocytosed, the macrophage uses

“frustrated phagocytosis” which results in necrosis of the target cell and inflammation [9, 23].

1.2.1.3. NK cells

NK cells are large granular lymphocytes containing perforin and granzymes in their cytoplasm, they are non-specific, and are involved in killing virally-infected cells and tumour cells [20]. They recognize their target cells in two ways: first, by linking to target cells via IgG antibodies that bind to Fc receptors on their surface (known as antibody-dependent cellular cytotoxicity). The second way is dependent on activating and inhibitory receptors expressed on their surface; the killer-activating receptors bind to various molecules, such as carbohydrate ligands, on other cell surfaces and stimulate the killing process. Also, all nucleated cells have MHC class I molecules which bind to the killer-inhibitory receptors on the NK cell surface and inhibit cytotoxicity. NK cells can distinguish between infected and uninfected cells by the alteration and down of regulation of MHC class I molecules on some infected cells, and also on some cancer cells [24]. When NK cells are activated, they release perforins and granzymes that cause target cell apoptosis in a similar way to Tc cells. Also, NK cells can use Fas/Fas-ligand binding mechanisms to kill tumour and viral infected cells by apoptosis [9, 23].

1.2.2. Humoral-immune response (B-cells)

The human body produces millions of clones of B cells that circulate in the blood and lymphatic system. B cells are created in the bone marrow and when they mature to express an IgM^+ receptor on their surface they migrate to secondary lymphoid tissues, such as spleen and lymph nodes. Each B cell surface has a unique surface immunoglobulin (sIg) that recognizes and binds a specific antigen, which causes B cell activation, proliferation and maturation leading to secretion of free antibodies, which contain binding sites typical of those in the original sIg. Some of the B cells become memory cells that can identify the same antigen epitope when the body faces the same antigen, and therefore the humoral immune response will be more rapid and efficient in attacking the pathogens. The main functions of B cells are to produce antibodies, act as APCs, develop into memory B cells after interaction with an antigen, and release cytokines as signalling molecules to help to regulate the immune response.

The development of a B cell occurs in several steps, each step is characterised by alterations in the content of the genome at the antibody loci. Antibodies are made up of two identical light (L) chains and two identical heavy (H) chains, and the genes identifying them are located in the Variable (V) and Constant (C) regions (Figure 1.4) [12, 25] .

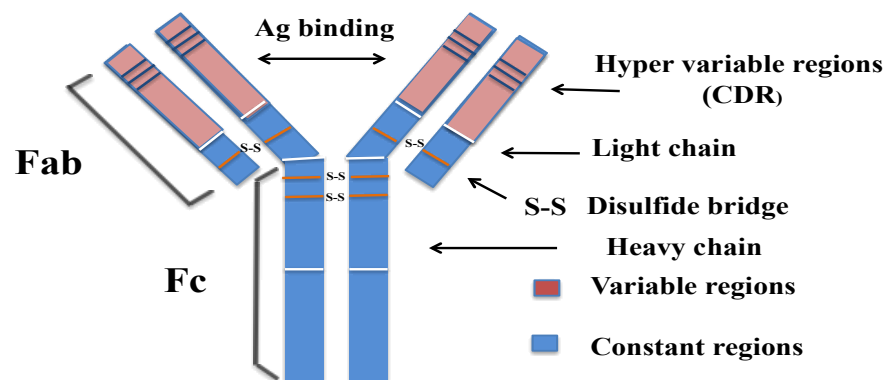


Figure 1.4: Antibody structure.

The antibody structure consists of two identical heavy chains, two identical light chains and has disulphide bridges between the chains. Heavy chains contain one variable domain and 3-4 constant domains that define the Ig isotypes, where γ (IgG), α (IgA) and δ (IgD) contain three constant domains, and the heavy chains μ (IgM) and ϵ (IgE) contain four constant domains. In mammals, there are only two types of light chain, λ and κ . A light chain contains two domains: one constant domain and one variable domain. The fragment crystallisable region (Fc region) is the part that combines with Fc receptors on cell surfaces. The Fc fragment is crucial for Fc receptor-mediated activity. The Fab region (fragment antigen binding) contains the variable domains that have hyper-variable regions: these have a high ratio of different amino acids within immunoglobulins specific for different antigen epitopes.

1.2.2.1. Sub-populations of B cells

1.2.2.1.1. B-1 cells

These are the first B cells that are created in the foetus, and in adults they are found mainly in the peritoneal and pleural cavities [26]. B-1 cells have been shown to work in the innate response; B-1 lymphocytes express (poly-specific) IgM in larger concentrations than IgG, they express little or no IgD, and they express CD5 protein, which binds to CD27 to facilitate B cell-B cell interactions. B-1 cells react weakly to protein antigens and more strongly to carbohydrates, there is no evidence for class switch and they secrete abundant antibodies of low affinity [12, 27].

1.2.2.1.2. B-2 cells

These are formed in the re-population that happens in bone marrow. They express IgD as well as IgM on their surface, undergo class switching and have a greater affinity to antigens than B-1 cells [12, 28].

1.2.2.1.3. MZ B cells

These exist in the marginal zones of the spleen, in the subscapular sinus of lymph nodes, in the epithelium of tonsils and in Peyer's patches. They have sIg and are implicated in innate/early adaptive immunity [12, 29-31].

1.2.2.1.4. Naïve B cells

Naïve B cells express IgM and IgD. They are activated by two mechanisms, the first is dependent mainly on Th cells (T cell-dependent activation), and the second is not dependent on T-cells (T cell-independent activation) [12, 30, 32].

1.2.2.1.5. FO B cells

Follicular (FO) B cells exist mainly in the germinal centre of lymph nodes. They are activated when they are exposed to Toll-like receptor (TLR) ligands and differentiate into short-lived plasma cells [12, 30].

1.2.2.1.6. Memory B lymphocytes

These cells are generated in lymphoid tissues after the activation and proliferation of B cells and they are located in the bone marrow, lymph nodes and spleen. They are long-lived, quickly stimulated and are prepared for a rapid reaction to a *repeated* exposure to an antigen [12, 30].

1.2.2.1.7. Plasma cells

These are terminally differentiated B cells that produce large amounts of antibodies against a specific antigen; they can be short-lived or long-lived [12, 31].

1.2.2.2. Stages of B cell development and differentiation

Before birth, B cell development takes place in the yolk sac, the foetal liver and foetal bone marrow, while after birth this occurs solely in the bone marrow. Differentiation of B cells can be classified into two phases, antigen independent differentiation and antigen dependent differentiation [31] (Figure 1.5).

1.2.2.2.1. Phase I: Antigen independent differentiation

In the bone marrow, the progenitor B cells (pro-B cell) express the adhesion molecule VLA-4, which contacts directly with the VCAM-1 ligand on stromal cells. This binding is used as a trigger for the production of IL7 from stromal cells that binds with IL7 receptors on the pro-B cells, inducing their development into precursor-B cells (pre-B cell) that then undergo proliferation. After proliferation the Ig genes are rearranged in an ordered fashion to generate the primary Ig repertoire [31, 33].

In the Pro-B cell phase, the heavy chain diversity (D) and joining (J) gene segments undergo DJ gene arrangements followed by V region and VDH creation. Later, these cells differentiate into pre-B cells by completing the light chain gene rearrangements. The pre-B cells can then express membrane Ig with a surrogate light chain, which consists of V pre-B as a variable region and lambda 5 ($\lambda 5$) instead of a constant region, and these become associated with Ig- α / Ig- β heterodimers to form a pre-BCR (pre-B-cell receptors). These changes mean that pre-B cells produce IgM (μ chains), which are transiently expressed in association with two smaller polypeptide chains (V pre-B variable and $\lambda 5$) (Figure 1.6) [34, 35].

Multiple cell divisions occur which results in the production of immature B cells that contains functional BCR (immature naïve B cell). In these cells the variable region of the heavy chain consists of three segments, V, D, and J, and they are recombined randomly through the VDJ recombination process. Similar processes occur in the light chain, but there are only two segments at variable region V and J [35]. The formation of the BCR starts when the recombinase enzyme complex catalyses the fusion of one DH region gene to a JH region gene with the deletion of

the intermediate DNA sequences. The recombinase then joins one VH region gene to the rearranged DHJH gene. The enzyme terminal deoxynucleotidyl transferase (TdT) is expressed, adding random nucleotides to the sites of VHDHJH joining and enhancing the diversity of amino acid sequences. That pairs with an L chain and produces IgM. When the VHDHJH element is followed downstream by exons encoding the C region for the δ chain, it produces IgD. These events occur because of alternative RNA splicing. Finally, if the rearrangement of VH, DH, and JH elements yields an H chain transcript and encodes a functional H chain protein, this H chain is synthesized and pairs with 2 proteins (called $\lambda 5$ and VpreB), which act as a surrogate light chain, and results in the expression of a pre-BCR. Once a functional heavy chain is produced, the cell down-regulates the gene and initiates an L chain rearrangement. It first begins with a κ element and, if this rearrangement is unsuccessful, continues with a λ element. A $V\kappa$ element rearranges to a $J\kappa$ element and produces a light chain, which, if it is functional, pairs with the H chain to make an immunoglobulin protein [36].

During maturation, if a B cell fails in any step it will die by apoptosis. Immature B cells leave the bone marrow and migrate to the spleen, where they complete their early development by differentiating into naïve mature B cells, which can be detected by the co-expression of IgD and IgM on the cell surface, follicular, or MZ B cells (Figure 1.7, 1.8) [12, 37]. This differentiation mechanism has been shown to include several enzymes and transcriptional factors.

After differentiation, around 10 % of the B cells are lost and the remaining 90 %, which are mature B cells, migrate into secondary lymphoid organs where they become functional, with a half-life of between 3 days to 8 weeks, and they die by apoptosis in the absence of antigens [31, 37, 38].

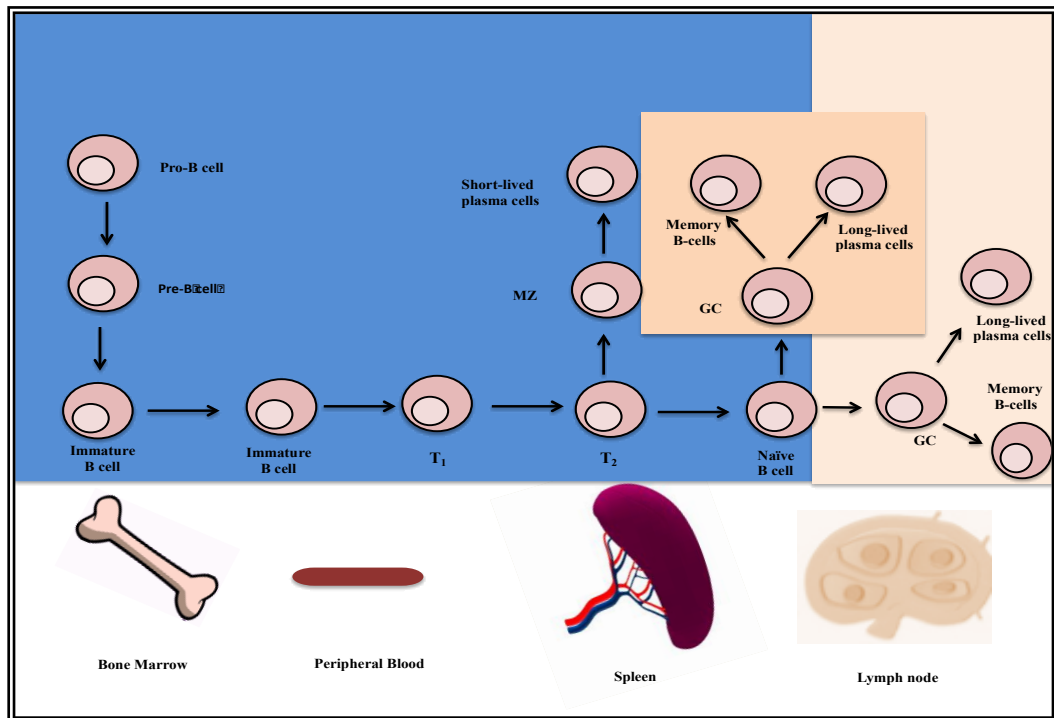


Figure 1.5: B cell differentiation in the bone marrow (BM) and periphery occurs in multiple steps.

The antigen-independent steps are in the blue background and the antigen dependent steps are in the cream background. In BM, the primary phases of B cell development result in the formation of immature B cells, which leave the BM and move to the spleen. These cells are now called transitional 1 (T₁) cells, which differentiate into transitional 2 (T₂) cells, and can continue their maturation to naïve or marginal zone (MZ) B cells. Naïve B cells leave the spleen and recirculate via secondary lymphoid organs and the blood. In the presence of an antigen, naïve B cells are activated and form germinal centres (GC). Cells with a high ability to bind to antigens are incorporated into the long-lived peripheral lymphocyte pool and are chosen to differentiate to memory B cells or long-lived plasma cells.

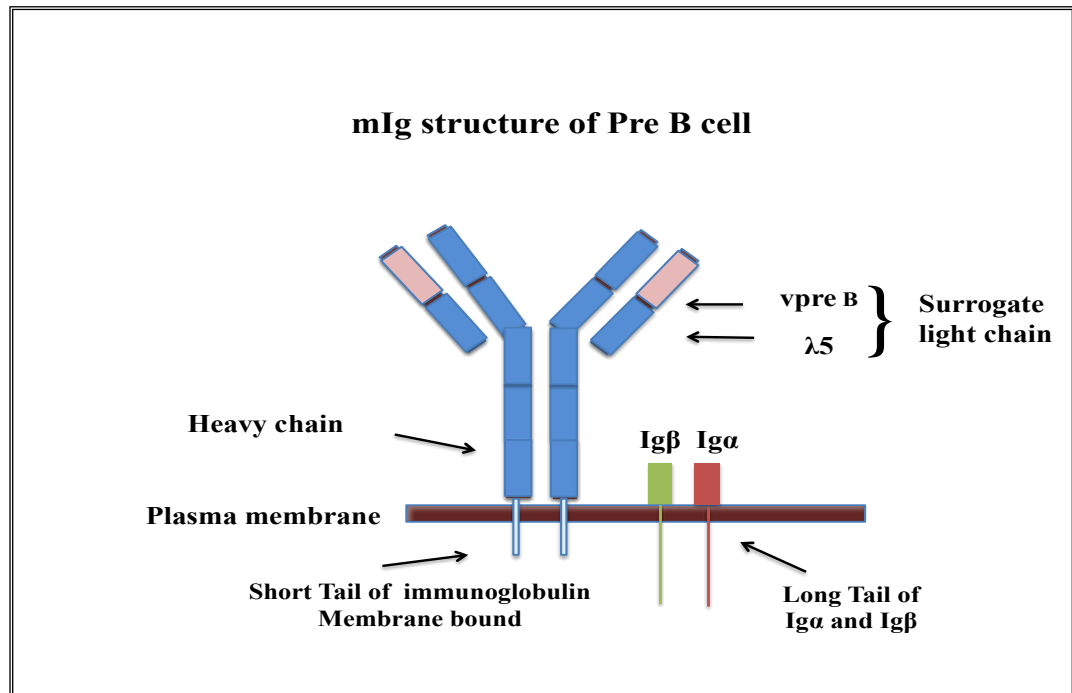


Figure 1.6: Membrane Ig in pre-B cells.

Pre-B cells have a membrane Ig (mIg), which contains V pre-B as a variable region that is combined with surrogate light chain, containing lambda 5 ($\lambda 5$) as a constant region. The mIg are associated with heterodimer Ig- α /Ig- β .

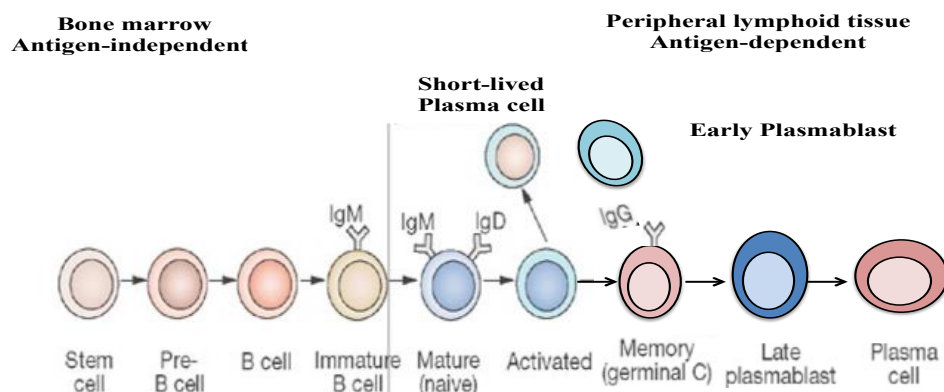


Figure 1.7: Stages of B cell differentiation.

The co-expression of IgD and IgM on the cell surface identifies the naïve mature B cells and, in the germinal centre, B cells could express IgG or IgA or IgE depending on the Ag type and type of T cell help received (This figure has been adapted from reference number [39]).

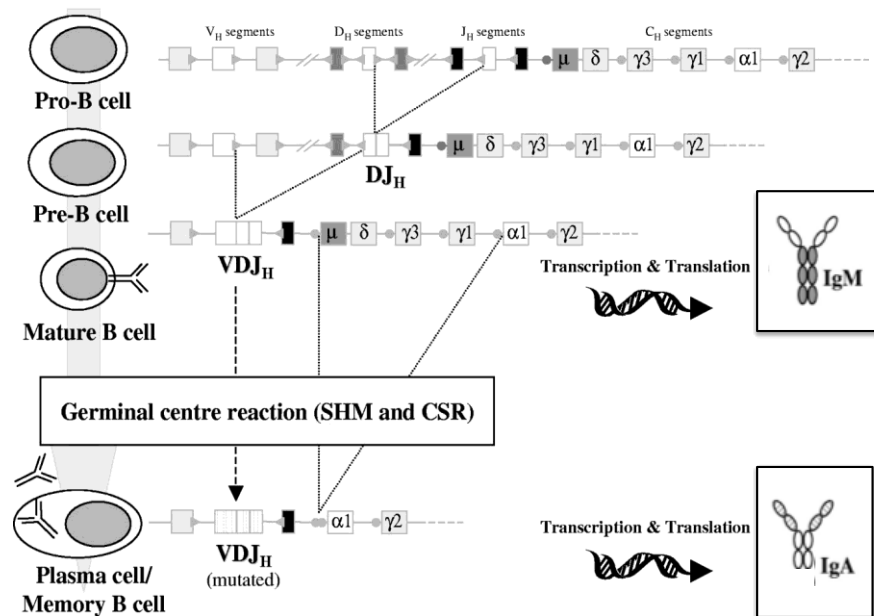


Figure 1. 8: B-cell development.

At the pro-B stage D_H join to J_H , creating a DJ_H rearrangement. Then, at the pre-B stage, V_H joins to DJ_H which results in production of the complete VDJ_H rearrangement. If this VDJ_H is functional, the cell can develop into a mature B-cell that expresses surface IgM (IgM^+). These cells are then able to enter the GCs where SHM and CSR occur, thus the cells become V_H - mutated, and class-switched (e.g., IgA) memory B or plasma cells (This figure has been adapted from reference number [37]).

1.2.2.2.2. Phase II: Antigen dependent differentiation

In the secondary lymphoid organs, further differentiation of mature B cells occurs dependent on its exposure to an antigen. The activated-mature B cells migrate into the primary follicles of lymphatic tissues and ultimately mature into F0 or MZ B cells. The cell-cell interactions need the expression of membrane-bound $LT\alpha/\beta$ trimers and TNF, while T cell-dependent B cell differentiation needs the recruitment of CD40 (TNFRSF5) by CD40L on activated $CD4^+$ T cells [12, 40]. CD30 (TNFRSF8) is expressed on activated B cells and has been found to be vital for effective memory B cell production, and CD27 is also involved in B cell memory development. After proliferation of B cells in germinal centres, the

primary immune response is mediated by short-lived plasma cells and long-lived plasma cells, and memory cells are generated for the secondary response [12, 25]. The specific mature B cells transform into plasma cells within 5 days. During this process, there is a progressive development of the ribosome and ER, where the content of RNA increases in the plasma cell that induces the protein synthesizing machinery and stimulates ER cisternae to become filled with antibody molecules, so the plasma cell can rapidly secrete them.

The formation of antibodies is consistent with the theory of clonal selection, which was proposed by Nobel Laureates Frank MacFarlane Burnet and Peter Medawar [41]. Lymphoid organs generate many B cells with different surface receptors, and the basis of the theory depends on a particular antigen epitope binding with the appropriate B-cell receptor (BCR), leading to the activation of a selected single B cell, which becomes an enlarged immunoblast cell and undergoes division and clonal selection and, therefore, many clones of activated plasma cells are produced [12].

1.2.2.3. B cell transcription factors

Development of the B cell lineage is dependent on bone marrow stromal cells, which generate IL-7 and many other factors such as PU-1, E2A, early B cell factor 1, paired box gene 5 (PAX), and interferon regulatory factor 8 (IRF8). *Pax5* is one of the most critical transcription factors as it restricts the developmental potential of lymphoid progenitors to the B cell pathway through blocking B-lineage-inappropriate genes, and it simultaneously stimulates B cell development through upregulating B-lymphoid-specific genes. Studies have shown that *Pax5* regulates gene transcription via chromatin remodelling, histone modification, and recruiting basal transcription factor complexes to their target genes [39]. Moreover, *Pax5* contributes to the variety of the antibody repertoire through regulating V_H-DJ_H recombination and it stimulates the contraction of the Ig heavy-chain locus in pro-B cells. Also, essentially all mature B cell types depend on *Pax5* for their differentiation and function, and therefore *Pax5* regulates the identity of B lymphocytes through B cell development. Accordingly, conditional damage of *Pax5* permits mature B cells from peripheral lymphoid organs to develop into functional T cells in the thymus via differentiation to uncommitted progenitors in

the bone marrow. *Pax5* has also been associated with certain diseases involving human B cell malignancies [25].

1.2.2.4. Surface markers

In the pro-B cell period, the B cells express CD45R, Ig- α / Ig- β , CD19, CD43 and CD24 and c-kit surface molecules. In the pre-B cell period, B cells express the same molecules, except c-kit and CD43, and they start to express CD25, and the α -chain of IL2 receptor [38, 42, 43].

1.2.2.5. Activation of B cells

The activation of B cell starts when the receptor recognizes and binds to an antigen. Activation of B cells occurs through two pathways, T-cell independent activation in response to carbohydrate or lipid antigens, and T-cell dependent activation in response to protein antigens (Figure 1.2) [11].

1.2.2.5.1. T-cell independent activation

The T-cell independent antigens can activate B cells without T-cell help; in the case of TI-1 type antigens, such as LPS (lipopolysaccharide), the first signal occurs through binding between BCR and antigens, and the second signal is produced by Toll-like receptors (TLR4). For the TI-2 type antigens, carbohydrates on the surface of bacteria have repeated epitopes that can react with, and crosslink, a large number of BCRs; the B cells can then proliferate without Th cells. Since there is no CD40 signal or cytokines involved, the antibody response is weak, the cells cannot undergo isotype switching, and they induce poor B cell memory [11, 12].

1.2.2.5.2. T cell dependent activation

T cell dependent activation of B cells needs two signals; the first occurs when an antigen binds and produces clustering of the BCR. The second signal occurs when the CD40L protein on the surface of an activated T helper cell binds the CD40 protein on B cell surface. Once a B cell binds the antigen, the antigen molecule is taken into the cell through endocytosis and is then represented on the cell surface by MHC class II molecules. This process means the B cell is sensitized and can bind with a Th cell. The Th cell is activated through binding to antigen-MHC class II complexes on the surface of the B cell, and this results in cytokine secretion, which stimulates the B cell to divide and produce thousands of identical plasma or

memory B cells. These plasma cells are responsible for secreting antibodies against these antigens, while the memory B cells stay inactive at this time, and later when the body is re-exposed to the same antigen, they will divide into plasma cells which are able rapidly to secrete antibodies [11].

1.2.2.6. Role of cytokines in B cell maturation and activation

The cytokine IL-7 is important in the development of B cells; it conducts numerous significant functions including assisting in the specification and commitment of cells to the B cell lineage, the proliferation of B cell progenitors, and maturation of pro-B to pre-B cells. During B lymphopoiesis, the regulation and modulation of the IL-7 receptor (IL-7R) signalling is critical as extreme or poor IL-7R signalling causes unusual or reduced development of B cells [44]. Studies have shown that IL-7 works with the transcription factors E2A, EBF, Pax-5, and others to control B cell commitment. IL-7 also works to control Ig rearrangement by modifying FoxO protein activation and *Rag* enhancer activity. Therefore, the appropriate IL-7 signals are transmitted to permit the commitment and development of B cells [44]. CD4⁺ T cells also secrete cytokines that are responsible for controlling B cell proliferation and differentiation. In delayed-type hypersensitivity reactions and cell-mediated immunity, naïve CD4⁺ T cells, in the presence of IL-12 and IFN- γ , develop into the Th1 subset. Subsequently, the Th1 cells stimulate the activation of the cell-mediated immune response, and therefore, CD8⁺ T cells, NK cells and macrophages are activated. During the detection and destruction of certain viruses, extracellular bacteria, soluble toxins, multicellular parasites and in allergies, Th2 cells can produce type-2 cytokines (IL-13, IL-4, IL-5, IL-10). Studies have shown that IL-2 and IL-4 producing cells activate B cells toward entry into M phase, and IL-4, IL-5, IL-6, and IL-13 signal B-cells to divide, differentiate and secrete antibodies. IL-4 and IL-10 stimulate the humoral response; they stimulate the growth and activation of mast cells and eosinophils, and switch antibody synthesis to IgE. Moreover, they suppress the activation of macrophages, T-cell proliferation, and the production of pro-inflammatory cytokines; they are also known as anti-inflammatory cytokines. Studies have also shown that Th1 cytokines can suppress the function of the Th2 subset. The type of cytokines that are produced from activated T cells can determine antibody class switching: for example, if a Th cell

produces IL4, then the B cell will switch to IgE production, whereas if IFN- γ is produced, then the B cell will switch to IgG production [12, 45, 46].

1.2.2.7. B cell class switching

Naïve B cells express IgM in a cell surface bound form; after maturation it can express both IgM and IgD, and engagement with antigens by sIg initiates the cell to differentiate into cells that produce antibodies, which are called plasma cells. If an antigen activates a B cell and there is also stimulation of CD40 by CD40-ligand on a helper T cell, the B cell class switching starts to produce different isotypes of the antibodies. During the Ig class switching, the change occurs in the constant region of the heavy chain, while the variable regions remain the same, and that does not affect the specificity for the antigen. These changes take place in the constant region genes on chromosome 14, where there is deletion of the μ -chain constant domain DNA and association with one of the other constant region genes, which produces other isotypes depending on the type of constant region added. This process enables a B cell to produce different isotypes of antibodies necessary to fight the relevant antigens [25, 31].

1.2.2.8. Antigen-antibody binding

The microbes involved in attacking the body usually contain multiple small or large antigens on their surface; the area where this antigen is bound to an antibody is called an epitope or an antigenic determinant, and the corresponding area on the antibody is called an antigen binding site or paratope. The folding of heavy and light chain complementarity determining regions (CDRs) within the variable regions creates the specific antigen-binding site; an antigen-binding site is formed from 6 CDRs, half of them are generated from the heavy chain and half of them from the light chain. The antibody specificity depends on the shape and the chemical composition of the amino acids that compose the CDRs, therefore the antigen epitope that has a complementary structure and chemistry can fit strongly with that site, binding with non-covalent bonds [11]. The two heavy and light chains of a single immunoglobulin are identical; therefore, they bind to identical antigen epitopes. The multiplicity of binding sites is called valency; IgG, IgE and IgD have a valency of two, secretory IgA has a valency of 4, and IgM has a valency of 10.

Each BCR has three components: a membrane immunoglobulin (mIg) associated with two accessory proteins, the heterodimers Ig α and Ig β . The C-terminus of the heavy chain that crosses into the cytoplasm of the cell is very short – only three amino acids in length – which is insufficient to activate a signalling response. The Fab region is extracellular and plays a role in ligand attachment, whereas the Fc region binds non-covalently with Ig α -Ig β , which have long cytoplasmic tails that consist of 61 and 48 amino acids, respectively. Both chains include an 18-residue long motif in their cytoplasm regions known as the immunoreceptor tyrosine based activation motif (ITAM); when an antigen binds to the BCR, the tyrosine residues in the ITAMs become phosphorylated, which means that certain SH₂ domains existing in different proteins can recognize them. Therefore, phosphorylated ITAMs have the ability to recruit molecules that include certain SH₂ domains to induce signalling (Figure 1.9) [47].

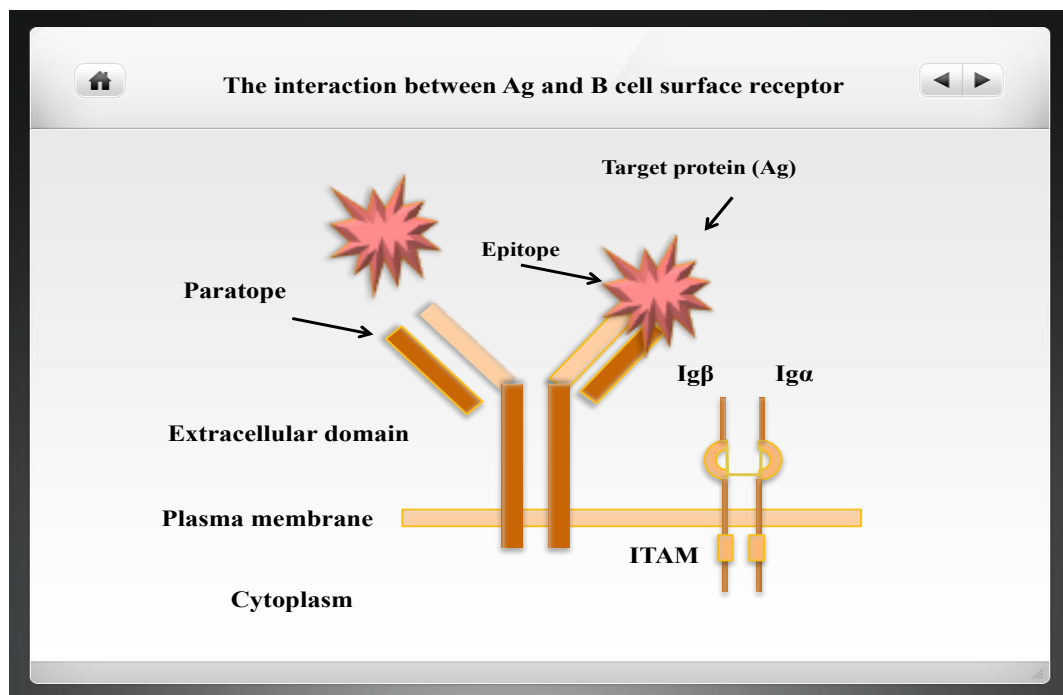


Figure 1.9: The interaction between Ag and B cell surface antigen receptor.

The antibody has a paratope site, which can interact with the specific antigen at the epitope site. ITAM become phosphorylated to induce signalling pathway activation; ITAM is immunoreceptor tyrosine based activation motif.

1.2.2.9. Immunoglobulins (Ig)

Secreted Igs are a group of glycoprotein molecules synthesised by B-lymphocytes and plasma cells and found in the serum, body fluids and tissues; they are also known as antibodies. When an antibody is digested with the enzyme papain, its molecular structure is divided into three parts, two identical parts called Fab fragments that contain the antigen-binding site and determine the specificity of antibody, and the third fragment is the Fc region, which stands for ‘fragment crystallisable’ because this region was found to form crystals when separated from the Fab regions. The Fc region determines all the biological functions of an antibody and determines its class. The antibody is composed of four polypeptide chains: two heavy chains and two light chains; the light and heavy chain loci are each composed of a sequence of V gene elements, followed by some D segments (for the heavy chain gene only), J segments, and C exons. Heavy chains (H) are made up from 4 segments (VH, D, JH, and CH), and light chains (L) are made from 3 segments (VL, JL, and CL) (Figure 1.10). The genes for the 9 different heavy chain types (IgM, IgD, IgG1, 2,3 and 4, IgA1 and 2, and IgE) are situated on chromosome 14 and those for the 2 light chain types (κ or λ) are located on chromosome 2 and 22, respectively [45].

Heavy chains consist of 440-550 amino acids whereas light chains consist of only 220 amino acid residues, each chain contains two types of domains – variable and constant domains. The H chain constant regions form the Fc domain of the molecule and are responsible for most of the effector functions of the Ig molecule (Figure 1.10). Both variable domains contain “hypervariable” regions or CDRs, which identify and bind to specific antigens. The type of heavy chain determines the immunoglobulin isotype, and based on the structural and antigenic properties, the Igs can be divided into five main classes, IgM, IgG, IgA, IgE and IgD. IgG and IgA classes can be further divided into subclasses based on differences in their amino acid sequences in the H chains, but the carboxyl terminal portions are constant in each subclass of antibody [11, 48]. Overall the different structures of these Igs give them different properties (Table 1.2).

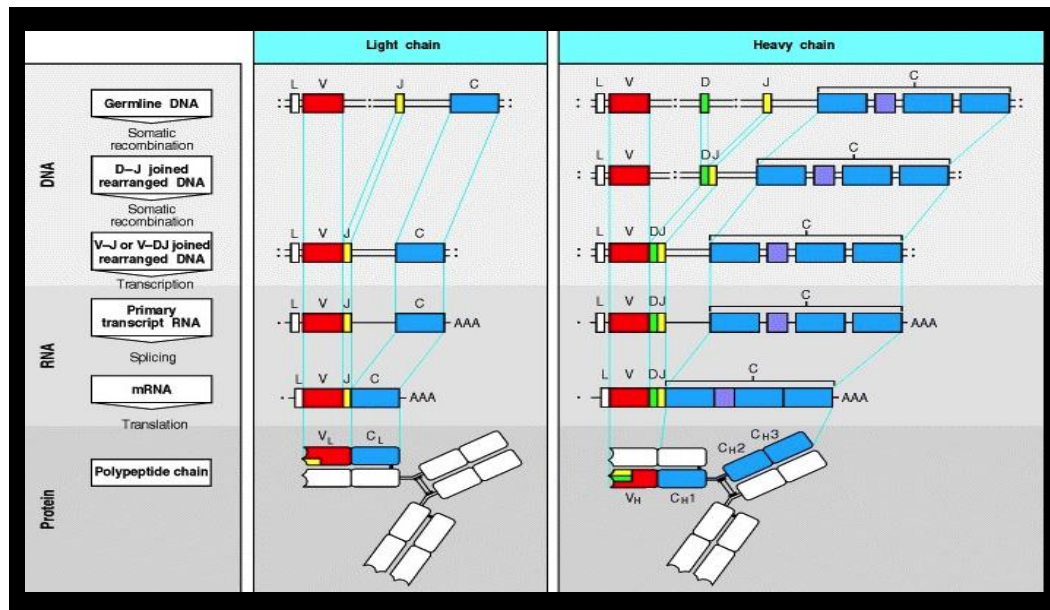


Figure 1.10: Schematic illustration of the immunoglobulins H and L chain modules.

Each light and heavy chain locus consists of a series of V (variable) gene elements, followed by several D (diversity) segments (for the heavy chain gene only), some J (joining) segments, and C (constant region) exons. H chains are composed of 4 segments (V_H, D, J_H, and C_H); L chains are composed of 3 segments (V_L, J_L, and C_L) (This figure has been reproduced from reference number [36]).

1.2.2.9.1. Immunoglobulin G (IgG)

IgG contains gamma heavy chains, and it is the most abundant antibody in the blood as it accounts for 75 % of the total serum antibodies. Based on the structural, antigenic and functional differences in the heavy chain constant regions – C_{H1}, C_{H2}, C_{H3} and particularly the hinge region – IgG is divided into four subclasses IgG 1, 2, 3, and 4, with IgG1 being the most abundant [49]. IgG is a monomeric antibody that contains two antigen binding sites and it is particularly important in secondary immune responses. It can cross the placenta from the maternal blood and provide the foetus with immunity. Due to differences in the CH domain, there are differences in flexibility and functional activity between IgG subclasses. Generally, IgG1 and IgG3 are produced in response to protein antigens, whereas polysaccharide antigens induce IgG2 and IgG4. In addition, IgG contributes directly to an immune response as in neutralization of toxins and viruses, and triggering the complement cascade is the main way to clear the opsonized

pathogens [49]. There are two ways for IgG to interact with the immune cells; either through direct cytophilic interaction with macrophages, which is independent of the F(ab) region of the antibody and requires only Fc interactions. The other mechanism involves specific Fc receptors (FcγRs), which trigger effector responses such as macrophage phagocytosis, NK cell ADCC, and neutrophil activation. FcγRs are characterised by the existence of an ITAM motif, either intrinsic to the receptor, as in the case of the human FcγRIIA (a receptor not found in the mouse), or more frequently, as part of a subunit associated with the γ chain, as in FcγRI and FcγRIIIA, receptors preserved between mouse and human. Moreover, human neutrophils have a specific receptor, FcγRIIIB that binds IgG immune complexes without generating activation. Cross-linking of the ligand binding extracellular domain leads to tyrosine phosphorylation of the ITAM by members of the src kinase family, followed by recruitment of SH2 containing signalling molecules, which interact the phosphorylated ITAM, most particularly the syk kinase family of molecules. The cell type which is activated by the Fc receptor determines the type of kinases that are involved in these signalling pathways [50] .

1.2.2.9.2. Immunoglobulin M (IgM)

IgM contains mu heavy chains, it forms a pentamer with a polypeptide chain called the J chain, and it contains 10 antigen-binding sites. It is the first antibody formed during the immune response, and it is a monomer when it acts as an antigen receptor on the surface of B-cells [41]. IgM accounts for approximately 5-10 % of the total serum antibodies and is located mainly in the bloodstream rather than other fluids. IgM is produced in the primary immune response and is commonly used to diagnose acute exposure to a pathogen. It not only participates in the first line of defence, but also plays an important role in immune regulation [49, 51]. The pentamer structure helps IgM to opsonize antigens for destruction and for fixation of complement.

1.2.2.9.3. Immunoglobulin A (IgA)

IgA contains alpha heavy chains, and constitutes about 10-15 % of serum antibodies, but it is highly expressed in body secretions such as saliva, mucus, tears, gastrointestinal, respiratory and genitourinary tracts and milk where it is called secretory IgA (sIgA); IgA makes up to 50 % of total protein in colostrum “first milk

for neonates”. In serum, IgA is a monomer and its levels tend to be higher than the IgM levels, but lower than IgG levels. However, at mucosal surfaces and in secretions, IgA levels are much higher than IgG levels, and the IgA has a dimer structure where it binds to a J-chain and another polypeptide chain called a secretory piece (derived by cleavage of the poly-Ig receptor that transports IgA across mucosal epithelial cells) [42]. Thus, IgA has an important role in protecting internal body surfaces that are exposed to the environment, where it blocks the attachment of bacteria or viruses to mucous membranes. IgA is classified into two subclasses according to differences in the structure and mainly in hinge regions, IgA1 and IgA2; the hinge region in IgA1 is longer than that in IgA2, therefore IgA1 is more sensitive to bacterial proteases, thus IgA2 predominates in several mucosal secretions. IgA is critical for protecting mucosal surfaces by attacking bacteria and viruses [49].

IgA interacts with a variety of receptors existent on different cell types and produces different outcomes. The poly-Ig receptor (pIgR) plays an important role on epithelial cells to transport IgA into mucosal secretions, as discussed previously. Another main receptor is Fc α RI, which has a variety of roles to assist in removing pathogenic attackers. All these receptors demonstrate specificity for the α -heavy chain of IgA, while some, e.g. pIgR and Fc α / μ R, also associate with other immunoglobulin isotypes. Fc α RI, is a member of a family of Fc receptors included within the much bigger immunoglobulin gene superfamily [52, 53], but it has vital differences from the other Fc receptors in this group. Its gene is located in the leukocyte receptor cluster near to the killer cell immunoglobulin-like receptors and leukocyte immunoglobulin-like receptors on chromosome 19, contrasting to IgG-specific Fc γ R and the IgE-specific Fc ϵ RI, which are clustered on chromosome 1. It is expressed on neutrophils, monocytes, eosinophils, platelets and some macrophages and dendritic cells. In addition, it is expressed on Kupffer cells in the liver where it has a role in mediating phagocytosis of pathogens that are present in the blood and have been opsonised by serum IgA [54]. Moreover, human Fc α RI binds equally well to human IgA1 and IgA2 – both serum IgA and secretory IgA – although the effects may be different. Fc α RI is a transmembrane glycoprotein with two immunoglobulin-like extracellular domains and a short cytoplasmic region that lacks any known signalling motifs, it associates with FcR- γ chain; also, some Fc α RI

do not associate with FcR- γ chain dimer but are still able to bind IgA and endocytose IgA immune complexes [52, 55]. There are other receptors such as Fc α / μ R, which interacts only with polymeric forms of IgA and IgM [52, 56]; and transferrin receptor (TfR) also can bind with IgA.

1.2.2.9.4. Immunoglobulin D (IgD)

IgD contains delta heavy chains, constitutes only 0.2 % of serum antibodies, and it is found on the surface of B-lymphocytes along with monomeric IgM, where it can act as a B-cell receptor that may regulate the activation and suppression of B-lymphocytes. It has been found that during B-cell ontogeny, IgM and IgD are the first antibody isotypes expressed during the primary adaptive immune response, and after leaving the bone marrow B cells express surface IgM and IgD with the same specificity, but they are created through different splicing of a pre-messenger RNA. In humans, some B-cells class-switch from IgM to IgD, and in the case of IgM deficiency, IgD can be used to replace its function [57, 58].

IgD is found in serum, and in human secretions such as human nasal and cerebrospinal fluids, and also in the amniotic fluid of pregnant women where its concentration gradually increases during the first half of gestation [58-60]. IgD is often observed as a predominantly mucosal Ig isotype due to the fact that the upper aero-digestive mucosa is a better localization of IgD-producing B cells. Also, the presence of IgD, as well as IgD producing B-cells, in the circulation and in upper aero-digestive mucosa-associated lymphoid tissues (MALTs) indicates that IgD undertakes its immunological functions systemically and in mucosal areas. Secreted IgD can attack many pathogenic microorganisms in the upper aero-digestive mucosa; studies have shown that, during various infections, the number of IgD-producing B cells is greatly increased in the upper aero-digestive mucosa in IgA deficient patients and serum IgD levels increase. Therefore, in IgA deficiency, secreted IgD may provide mucosal protection [58, 61]. Rogenine *et al.* showed that the fractional catalytic rate of IgD was similar to that of IgA, and much higher than those of IgM and IgG, which indicates that IgD might be catabolized in similar ways as IgA, but in very different ways from IgM and IgG [62].



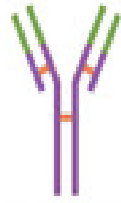
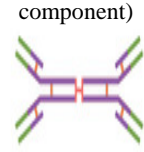
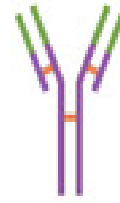
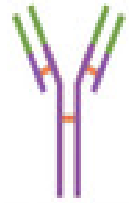
Cell-associated IgD includes transmembrane IgD, intracellular IgD and secreted IgD bound to various cell types; for example, crosslinking of IgD receptors on T-cells was observed as a preventive mechanism for T-cell apoptosis. Also, some

myeloid cell types are able to bind secreted IgD; studies showed that, in healthy subjects, basophils rather than neutrophils or eosinophils appeared to express more surface IgD [58], but under certain pathological condition, such as skin allergies and inflammation, neutrophils and/or eosinophils can bind significant amounts of IgD [58, 63]. Studies of monocytes did not show any significant IgD binding [64]. Furthermore, IgD can act as an immune-modulator that can promote the immune defence by activation of IgD-interacting cells in tissues, the production of activating factors consequently causes damage and inflammation of tissues. The levels of serum IgD increase in many chronic infections, which suggests there is an important role of IgD in these types of infections [58, 65].

1.2.2.9.5. Immunoglobulin E (IgE)

IgE contains epsilon heavy chains, it is a monomer, it has two antigen binding sites and it constitutes only about 0.002 % of the total serum antibodies. IgE binds strongly to basophils and mast cells via its Fc region, it is important during allergic responses, and it protects external mucosal surfaces by promoting inflammation that enables IgG, complement and leucocytes to enter the tissue, as well as causing removal of microbial toxins through coughing, sneezing and vomiting [61]. IgE is associated with hypersensitivity and allergic reactions, as well as the response to parasitic worm infections. It has high affinity to bind FcεRI, which is expressed on mast cells, basophils and eosinophils. Recently, anti-IgE antibodies (e.g. the humanised monoclonal antibody omalizumab) have been used to treat allergy and asthma; these antibodies bind to free IgE and B-cell surface IgE, but not IgE bound to FcεR since the latter could promote degranulation and inflammatory mediator release [49].

Table 1.2: Summary of the classes of immunoglobulins [66].

Characteristics	IgG	IgM	IgA(m)	IgA(d)	IgD	IgE
Structure	Monomer	Pentamer	Monomer	Dimer (with secretory component)	Monomer	Monomer
						
Percentage of total serum Antibody	80%	5-10 %	Represent 80% of total IgA	Represent 15% of total IgA	0.20%	0.05%
Location	Blood, Lymph, intestine	Blood, Lymph	Blood, Lymph,	Secretions (tears, saliva, mucus, milk, gastrointestinal, respiratory and genitourinary tracts)	Blood, Lymph	Bound to mast and basophil cells throughout body, blood
Half-Life in Serum	23 days	5 days	6 days	6 days	3 days	2 days
Complement-Fixation	Yes	Yes	No	No	No	No
Placental Transfer	Yes	No	No	No	No	No
Known Functions	Enhance Phagocytosis, neutralize toxins and viruses, protect foetus and new born	Effective against microorganisms and agglutinating antigens, first antibodies produce in response to initial infection	Shows a good response to proteins antigen and to lesser degree to lipopolysaccharides.	Localized protection on mucosal surface	Presence on B-cells functions in initiation of immune response, upper digestive tract	Allergic reaction, possibly damage to parasitic worms

1.2.2.10. Function of antibodies

The function of an antibody results in antigen neutralisation/destruction, and this can occur by various methods including agglutination, opsonization, antibody dependent cell mediated cytotoxicity (ADCC), complement mediated lysis and inflammation, and neutralization [12, 67].

1.2.2.10.1. Agglutination

This occurs when antibodies interact with particulate antigens and cause a clumping of particles. Smaller clumps are eliminated by phagocytosis, but large clumps may result in damage to tissues. IgM and IgG can work as agglutinins, but IgM is more efficient due to its pentameric structure [12, 67].

1.2.2.10.2. Opsonization

During opsonization, higher concentrations of IgG antibodies (opsonic antibody) are attached to larger antigens, and they bind with their Fc regions to several Fc receptors on the surface of macrophages and neutrophils. This binding induces a signal-transduction pathway that leads to the phagocytosis of antigen-antibody complex. The pathogen is then killed by various mechanisms, such as enzymatic digestion, oxidative damage and membrane disruption by antibacterial peptides [12, 67].

1.2.2.10.3. Antibody-dependent cellular cytotoxicity (ADCC)

Macrophages, neutrophils, eosinophils and NK cells have cytotoxic mechanism that destroy tumour cells and viral infected cells; their Fc receptors interact with IgG antibodies bound to the target cells and secrete specific components, such as lytic enzyme, perforin and TNF, which help to kill the target cell [12, 67].

1.2.2.10.4. Complement mediated lysis and inflammation

The formation of an antigen-antibody bond can activate blood complement proteins to bind to the antibody CH₂ domain of the Fc region, and this cascade of complement proteins results in membrane attack complex formation on the cell wall of pathogens, which produces a functional pore. Thus, the water and electrons of pathogenic cell are lost and this disturbs the osmotic stability of the cell. The complement cascade can also lead to phagocytosis by macrophages and neutrophils, and opsonisation, which can induce an inflammatory response [12, 67].

1.2.2.10.5. Neutralization

Studies have shown that antibodies can neutralize soluble toxins, which are secreted from viruses, bacteria or fungi and prevent them from damaging cells [12, 67].

1.3. Cigarette smoke and the immune system

Cigarette smoke (CS) has numerous toxic chemical constituents, which can be characterized as having cytotoxic, mutagenic, carcinogenic, and antigenic properties (Figure 1.11) [68, 69]. These toxic ingredients of CS quickly dissolve in the fluids of oral and airway epithelial linings, and are systemically taken up in either passive or active inhalation. They enter the body mainly through the pulmonary alveoli and are spread into all tissues by the blood. The immune protectors in the blood, such as B cells and T cells, can react to these stimuli. Chronic inhalation of CS has adverse effects on both the innate and adaptive immune systems, which consequently leads to an immunological impairment [4, 70].

In the 1960s, the effects of tobacco-smoke on immune and inflammatory processes were recognized for the first time. Studies of the mechanism of how CS impacts on the immune system have found that there are numerous toxic effects, such as carcinogenesis which occurs directly or indirectly with genetic or epigenetic effects and altered gene functions (DNA repair, and tumour suppressor genes), and CS can trigger multiple mechanisms to produce cancer and other diseases [4].

Molecular studies have shown that, in the presence of smoking components, several nicotinic receptors are up-regulated in B cells [71], and long-term exposure to nicotine can suppress B cells secretions, block the cell proliferation and development, and completely destroy the standard immune functions [72-75]. Therefore, all these results show that smoking causes abnormal changes in the immune system and produces diseases partly through the dysregulation and impairment of B cells [75-79]. Several studies have determined that smoking can enhance the incidence of COPD and emphysema [75, 80-82], asthma [83], chronic periodontitis [75, 84, 85], and inflammatory reactions, which highlights the importance of the effect of smoking on diseases [75, 86].

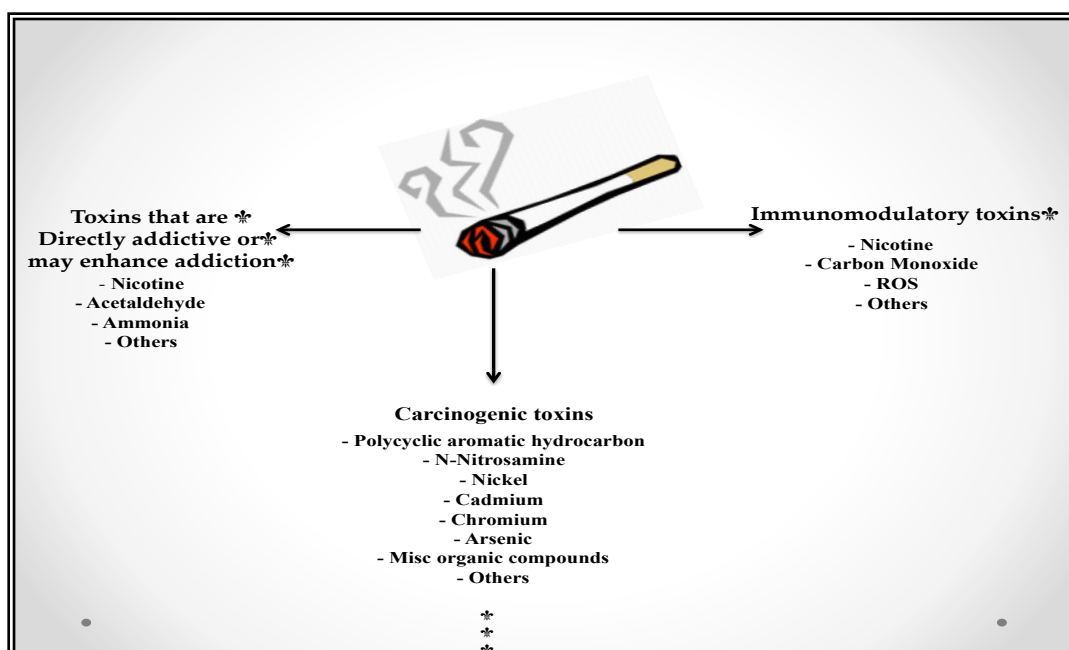


Figure 1.11: CS is a combination of thousands of chemical compounds, produced from the burning of tobacco.

These compounds are classified according to their known effects: 1) numerous carcinogenic toxins, 2) addictive CS toxins; although many chemicals contribute directly or indirectly to the addictive nature of CS, nicotine is the major one. 3) immunomodulatory factors such as nicotine, carbon monoxide, ROS, and acrolein [4].

1.3.1. Mechanisms of how cigarette smoke extracts exert inflammatory and suppressive effects on immune cells

Studies have shown that burning of tobacco is one of the important sources of many reactive oxidative substances (ROS) that do not exist in the raw tobacco leaf and are not trapped by cigarette butt filters [87]. The products of tobacco burning can be divided into gaseous and particulate constituents [68, 88]. ROS in the gaseous phase is short lived and mainly impacts the upper airways; whereas the particulate phase has highest concentration of toxic CS components and can generate more free radicals. In the mucosal surfaces lining the oral cavity, sinuses, and airways, these gaseous and particulate CS components start to react with the immune system [4, 87]. These ROS cause many harmful effects on epithelial cells lining the airways through stimulation of the peroxidation of lipids and other cell membrane parts, activation of oxidative-sensitive cellular pathways, and they can cause destruction

of DNA [4, 89]. ROS can also trigger epithelial cell intracellular signalling cascades, generating IL-8 and TNF α and resulting in stimulation of inflammatory genes [4, 90, 91]. The secretion of these inflammatory mediators leads to stimulation of chronic immune cell recruitment and inflammation.

CS can stimulate T-cells to proliferate and produce cytokines that mediate important biological functions; CS enables the generation of Th2 which stimulates the production of IL-4, and suppresses Th1 cells [4, 92-96]. Studies have found that there is inhibition of the activation of dendritic cells after severe exposure to cigarette smoke extract (CSE), which leads to decreased production of IL-12 (Th1 polarizing) and IL-23 (Th17 polarizing) cytokines [4, 93, 97]. Similar results have been observed in other studies which found, lower concentrations of IL-12 and IL-23 were secreted from lung and systemic dendritic cells isolated from mice who were exposed to CS [97]. These studies also found lung eosinophils numbers were higher in mice exposed to CS, which is a marker for Th2 inflammation, and decreased Th1 cytokines levels [4, 98]. Studies in animal models have shown CS increased Th2 polarized eosinophilic airway inflammation [96], which was similar to the results of *in vitro* stimulation of peripheral blood T-cells of individual smokers, as they formed higher concentrations of IL-13 (Th2 cytokine) compared to the non-smoker control group [4, 95]. Whether CS stimulates Th17 inflammation is not fully understood, although some studies suggesting that chronic CS exposure could stimulate Th17-mediated immunity [99] and increased the occurrence of inflammatory diseases that accompany Th17 inflammation [4, 100, 101]. The mechanisms by which CS activates Th2 and Th17 inflammation are yet to be fully elucidated, but they may include inhibition of Th1 polarization and affecting cytokine production [93], altered activation of antigen-presenting cells [96, 97], stimulation of Th2 polarization, effecting genetic factors, and probably causing direct effects on T-cells. The differences between the observations in these studies could be due to variations in the techniques used to extract CS, differences in the models of exposure, and additional co-factors [4, 94, 102].

1.3.2. Mechanism of how CS alters mucosal immunity

The airway epithelium protects the body from many invaders through the control of the immune system response. CS can immediately stimulate the airway epithelium, and this results in an immediate influence on chemokine and

inflammatory mediator secretion (Figure 1.12) [103-105]. This reaction is due to epithelial cells expressing TLRs which identify pathogen-associated molecules [105]. For instance, TLR3, which is expressed on airway epithelial cells, can combine with viral RNA to cause an excessive amount of chemokines to be secreted [4, 106]. Also, current or prior smokers, and acute pneumonia patients, have significantly decreased levels of beta-defensin-2 in their pharyngeal fluid and sputum [4, 107]. Studies have also shown that CS can greatly reduce mucosal ciliary motility and raise goblet cell numbers, which have a role in protecting mucous membranes, and it can also induce mucus hyper-secretion [4, 90]. These changes could possibly cause persistent stimulation of mucosal epithelium with reduced anti-microbial action in relation to the clearance of infection, and this could help to clarify the increased probability of smokers undergoing microbial colonization and infection.

The components of CS can trigger numerous cell-signalling pathways, affecting cell stimulation and regulation of inflammation, the cell cycle, and other regulatory genes. For example, studies have reported that CS can affect mitogen-activated protein kinases (MAPK), nuclear factor kappa-B (NF- κ B), signal transducer and activator of transcription (STAT), and activatory protein-1 (AP-1). NF- κ B plays a crucial role in controlling the immune response to infection, and improper control of NF- κ B can result in cancer and inflammation; STATs are a family of intracellular transcription factors that mediate various roles in cellular immunity, proliferation, and apoptosis, and AP-1 controls gene expression in response to various stimuli, such as cytokines, bacterial and viral infections [4, 97, 102, 108, 109]. Due to the effects of CS on NF- κ B and AP-1 transcription factors, it can lead to changes in chemokine production, corticosteroid resistance, responsiveness to acute pathogens, and altered cell death regulation [4, 109-112].

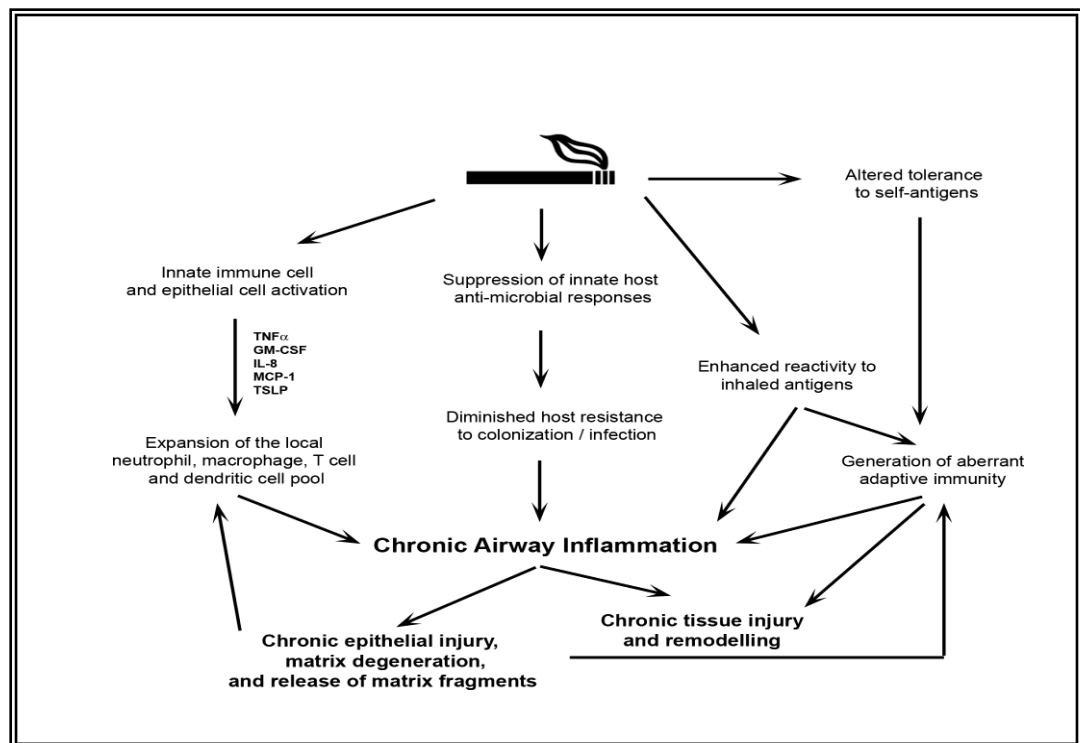


Figure 1.12: CS regulates inflammation and stimulates chronic inflammation in the airways by various mechanisms.

Directly by triggering epithelial and immune cells to stimulate the secretion of pro-inflammatory factors and promote the upregulation of other immune cells such as neutrophils, macrophages, T-cells, and dendritic cells. CS can also damage the innate host defence mechanisms, reduce the innate response to pathogens, and change the adaptive immune response to inhaled antigen, which leads to chronic injury and inflammation of the airways. Recurring injury combined with abnormal responses to self-antigens and co-existent pathogen colonization might increase the progress of autoimmunity, which further extends tissue damage and inflammation (This figure has been adopted from reference number [4]).

1.3.3. Cigarette smoke and innate immunity

The lungs are an important route for environmental pathogens and antigens to enter the body; in the respiratory tract, the mucociliary escalator of the large airways eliminates most of the inhaled external substances, and alveolar macrophages (AMs) and other monocytes play an important role in innate immunity in the lungs. CS is a significant risk factor for acute respiratory tract disease and COPD; studies have shown that CS increases the AM numbers by several fold, leading to increased

concentrations of lysosomal enzymes and secreted elastase, which may cause damage to connective tissue and parenchymal cells of the lung, contributing to COPD pathogenesis [113]. Moreover, AMs from smokers release significantly higher levels of reactive oxygen radicals and have higher myeloperoxidase activity, which are considered to be crucial mediators in the killing of intracellular pathogens, but in spite of this increased activity, AMs from smokers have a reduced ability to phagocytose and kill bacteria [5, 114]. In addition, several studies have established that smokers' AMs have impaired function and secrete lower amounts of cytokines, which are important in the regulation of the early immune response of the host to pathogens [115]. Experiments in animal models have shown that smoking causes various morphological, biochemical and enzymatic alterations in AMs that can damage the anti-bacterial defence and the inflammatory response in the lungs [116].

Comparisons between healthy non-smokers, ex-smokers with COPD, and current smokers show that the current smokers with or without COPD have the highest proportion of activated CD8⁺ T lymphocytes, NKT cells and NK cells in their peripheral blood. Therefore, these results suggest that smoking affects the activation of the systemic killer cells. Furthermore, there is a positive correlation between CD8⁺ T-lymphocyte and NK cell activation with the number of cigarettes that are currently smoked. However, there is no correlation between cell activation and lung function, so cell activation is more related to smoking habits than to disease progression. Overall these studies have suggested that systematic activation is related to smoking per se, while lung activation is related to COPD progression, and the activation of killer cells in COPD airways can play a role in COPD pathogenesis [117]. Similar studies have indicated that, in BALF, the numbers of CD8⁺CD25⁺ T-lymphocytes are significantly lower in ex-smokers compared with current smokers with COPD [118].

1.3.4. CS and adaptive immunity

CS increases the number of human blood leukocytes, but they have lower functional activity. Several investigations have shown that long-term smoking drastically decreases serum Ig levels in humans, and animal studies have shown that a consequence of exposure to CS is a significant reduction in the antibody response to variety of antigens, as well as increased autoantibody levels. This could explain

the higher susceptibility of smokers to certain autoimmune diseases, for example, rheumatoid arthritis [119]. Moreover, investigators have observed that T cells from smokers have a reduced ability to proliferate and show lower functional activity, suggesting a weakened immune response [5, 70]. Studies in Caucasians showed that smoking increased the numbers of peripheral-blood CD4⁺ cells, whereas smoking caused a decrease in T-cell numbers in African-Americans [120]. In COPD patients, the numbers of NK and NKT cells are increased in induced sputum but decreased in peripheral blood, while CD8⁺ T-lymphocytes are increased in both the peripheral airways and lower respiratory tract [121, 122]. In addition, experiments in animals have shown that CS that contains higher concentrations of nicotine and tar stimulate alterations in T-cell response more than smoke that contains lower levels of these components, suggesting these components play a key role in the observed changes after smoking [5, 70].

1.3.5. Effects of CS on Immunoglobulins

There are several studies that confirm that serum IgG, IgM and IgA levels are lower in smokers than in non-smokers by 10-20 % [123-125]. In terms of IgA, Barton *et al.* (1990) reported that, in healthy individuals, salivary IgA levels were lower in smokers than in non-smokers, whereas salivary IgM levels were higher in smokers, and the effects on IgA were reversed after stopping smoking. Similarly, smokers with head and neck tumours had lower salivary IgA and higher salivary IgM concentrations compared with non-smoking subjects. Overall this research provides evidence that there is an effect of smoking on mucosal immunity, which was confirmed by studies that, in a previous examination of salivary IgA in mixed unstimulated saliva, showed that tobacco smokers had decreased levels of salivary IgA when compared with the normal group [126, 127]. Decrease of secretory IgA in saliva could be caused by an impact on the salivary glands responsible for the formation of the secretory IgA or on the cells of the immunological system involved in the production of the IgA molecules [127, 128]. Conversely, Norhagen *et al.* (1998) reported that, in unstimulated saliva, immunocompetent smokers have higher levels of salivary IgA that may help in protection of oral mucosa, they showed there to be lower levels of IgG and IgM in stimulated saliva (i.e. induced by chewing paraffin wax) and they surmised that this may decrease inflammation

in oral mucosa [129]. Other research showed that serum IgA levels are higher in healthy smoking individuals compared with COPD smoking patients [130].

There is some conflicting evidence, however, that shows that there were no significant differences in salivary IgA, IgG, while IgM was significantly lower in smoking compared to non-smoking individuals [131], and Lie *et al.* (2002) report similar levels of salivary IgA between gingivitis trial smokers and non-smokers [132], which is supported by other studies that found no evidence of a significant difference in serum IgA levels between smoking and non-smoking individuals [133, 134]. Further contradictory evidence comes from studies using broncho-alveolar lavage fluid (BALF), some found reduced IgA levels in smoking individuals [135], whereas other studies found enhanced levels of IgA in the BALF, which may be due to differences in experimental set up, but overall these results suggests further study is required [136, 137].

In terms of IgM levels, some studies have shown that there are no significant differences in the levels in serum between smokers and non-smokers [138], but in saliva researchers have found significant increases in salivary IgM levels compared with non-smokers, and this is combined with low levels of salivary IgA. Overall there are more studies that indicate that, in stimulated saliva, individuals who have IgA immunodeficiency have increased IgM levels, which suggests there may be a measurable change [126, 129].

Studies of IgG indicate that healthy smokers have lower serum IgG levels than non-smoking individuals [133]. Other research showed that salivary IgG levels are the same between smoking and non-smoking individuals [139]; however others reported that salivary IgG levels are lower in smoking compared to non-smoking individuals but there is also evidence to suggest that tobacco smoking does not affect the compensatory rise in salivary IgG response over IgA deficiency in smoking and non-smoking individuals, all of which again suggests more research is needed in this area [126] [129, 140].

Some studies of salivary IgD have shown its levels do not have significant differences between smokers and non-smokers, but other research has found that in cigarette smokers the level of serum IgD is twice as high as in non-smokers, but this returns to normal after smoking ceases. Studies have also indicated that, in

COPD patients, the level of IgD in serum and nasal secretions is higher than in control individuals, which suggests that the reduced IgD levels may be related to progression of COPD [141].

Overall there are numerous studies on the consequences of inhalation of smoke on the human immune system and the results suggest that the qualitative and quantitative effects of CS on the immune system depend on a variety of factors including sex, duration of smoking, and ethnicity of the subjects studied. Although CS increases the risk of infections in the human body through diminished humoral and cell-mediated immunity, the findings vary depending on the conditions used in the research studies [5].

1.4. Protein microarray overview^[142]

Protein microarray is a relatively novel high-throughput technology used to detect the amount of an analyte in various samples including serum, saliva, and urine. Currently there are many different forms of microarray that are used for different purposes: for example, to detect many different substances, to measure gene expression at the mRNA or protein level, to discover mutations, for genotyping, to (re) sequence DNA, and to find chromosomal changes [142].

Protein microarray uses the same concept as the Enzyme-Linked Immuno-Sorbent Assay (ELISA) technique; it offers the possibility simultaneously to evaluate a variety of analytes across a broad spectrum of concentrations, using smaller volumes and at a lower cost than in the traditional method (ELISA). There are many different categories of microarrays (platforms), and they all require a high density and quantity of biomolecules fixed onto a specific surface. Generally, there are five essential steps in microarrays, the attachment of the biomolecules to a platform, preparing samples for detection, hybridization, scanning, and analysis of the data. Protein-antibody microarray is one of the most common protein microarrays; the principle of this array is that the capture antibodies are printed onto a specific slide, then the slide is incubated with the samples, followed by the detection antibody; this method can be used to detect proteins of interest in biological samples (Figure 1.13) [143-145].

Microarray has been used to offer novel insights into the pathogenesis of smoking-induced disease by examining specific samples, such as the progression of COPD

[146-148]. The major advantages of microarrays over ELISAs are microarrays require a smaller quantity of sample to run the assay, they have the capacity to detect more than one inflammatory mediator in one experiment, they can carry out many repeats of the same analyte in the same experimental conditions, and the possibility to detect a mixture of proteins across a range of concentrations. All of this leads to a reduction in time and cost to run the assay. Although there is evidence of the effectiveness of this technology, more effort on the reproducibility, and further validation and optimization is necessary before it will be widely accepted in medical research [143, 149, 150].

1.4.1. Microarray slide surfaces and immobilisation

The slide surfaces are one of the major sources of variation in development of reproducible protein microarrays; the types and chemistry of the slide surfaces determine the type of immobilisation between the slide and the protein that is printed on it [151, 152]. The printed proteins and the slide surface combine through different physical interactions, including Van der Waals, covalent, ionic or hydrophobic bonds, that need to be optimised according to the properties of the protein of interest, as these interactions should not alter the structure of the protein [151, 153]. There are many commercial slides available where the glass of the slide is 'silanized', which means the slides are covered in a silane solution, each of which has a special functional group. Amino-silane, epoxy-silane, hydrogel and aldehyde coatings are the most commonly used slide surface modifications, and each offers different properties [154].



Figure 1.13: Picture of the BioRobotics MicroGrid II Arrayer.

1.4.2. Spot size and morphology

Once the capture antibody or antigens have been printed on the slide surface, they can be seen as rounded spots on the slide [155]. The morphology and size of the spots depend on the slide surface chemistry and the printing buffer used; some slide surfaces can lower spot scattering, causing a reduced spot diameter and improved spot morphology [155]. Weak spot morphology can occur from a failure of the slide surface to combine with the functional antibodies, which eventually reduces signal intensities [156]. The printing buffer can also determine the size and number of spots on the surface of slides, which consequently affects the signal intensities of the spots. The size of the spots on the existing commercial microarrays have been calculated to provide the optimal probe/analyte capture efficiency (50 to 200 μm diameter), but any further reduction of the spot size to the nm scale may result in reduced efficiency, due to kinetic-limiting behaviour [157].

In current research, the silicon pins that have been used could pick up 0.1 μl (100nl), if they fill completely, assuming they are empty when filled. The way we use them is to print a set number of samples (but ensuring the pin does not run out) then refill and print again, until all spots of a particular sample are printed, then the pin is washed multiple times and is dried before moving to the next sample.

The numbers of spots produced by a pin full of liquid are dependent on many variables: humidity, the type of print solution, the surface, what is in the print

solution (protein / DNA /polysaccharides etc), dwell time (i.e. how long the pin touches the surface for each transfer, etc).

On an ideal setup, with a buffer that retains water well (i.e. reducing evaporation), with high humidity, using aminosilane or poly-L-lysine slides, the volume of each spot is approximately 500pl and, assuming a conservative 50 spots per fill of the pin, 20µl of a sample could print 75,000 spots (that would be about 292 x 64 pad arrays at 4 replicate spots per sample per array). However, evaporation loss from the plate has to be borne in mind, which can be substantial, especially on a slow print (i.e. with one pin), and this would lower the estimated number of spots that can be printed. Using several pins, and printing one array per slide, avoids much of this issue.

1.4.3. Spot background

The spot background is an expression used to identify the background signal that develops on the spot (or between the spots) in the absence of bound antigen; this can decrease the sensitivity of the assay, particularly at the lower end of the detection spectrum. The spot background is a result of non-specific binding of proteins to the slide which is affected by the slide surface chemistry; it can be caused by protein denaturation, which increases the possibility of non-specific binding [158].

1.4.4. Signal detection and generation

Fluorescence is one of the most common methods used for signal generation, and is produced when a substance absorbs electromagnetic radiation or light of a long wavelength (high energy), and then emits light of a shorter wavelength (lower energy). The excitation wavelength can be in the wavelengths visible to human eyes, or the UV region of the spectrum, and some fluorophores, for example Cy5, are excited by wavelengths around 625 nm and emit light in the red spectrum (532 – 670 nm) [159]. In microarrays, the detection of the signals depends on two different strategies, a label-free strategy such as surface Plasmon resonance (SPR), or labelled-probes which can be used directly, indirectly or in a sandwich assay [160]. The direct method uses labelled antibodies bound directly to the target molecule of interest; the benefit of this is that it allows all proteins to be labelled in the same way accurately but, conversely, it can cause a high background due to

non-specific adsorption of proteins and this may affect the sensitivity of the assay. The indirect method works by immobilizing the target antigens first using a primary antibody, and then a second labelled antibody is used for detection [161].

The most commonly used method in antigen-antibody microarrays in the literature is the fluorescence detection method, which includes using Alexa fluor, Oyster or fluorescent dyes such as Cyanine [162, 163].

An important part of the detection method is the amplification of the signal through the addition of biotin, alkaline phosphatase or tyramide signal amplification [164]. In this study, a tyramide amplification reagent was used; tyramide signal amplification is an enzyme-mediated detection technique that uses the catalytic activity of horseradish peroxidase (HRP) to produce high-density labelling of a target protein in a sample (Figure 1.14). This process occurs through attachment of a probe to the target, then secondary detection of the probe by an HRP-labelled antibody or streptavidin conjugate. The amplifier improves the sensitivity of immunoassays, including antigen microarrays, and studies have shown that the tyramide amplification system considerably improves the performance of antibody microarray assays [165].

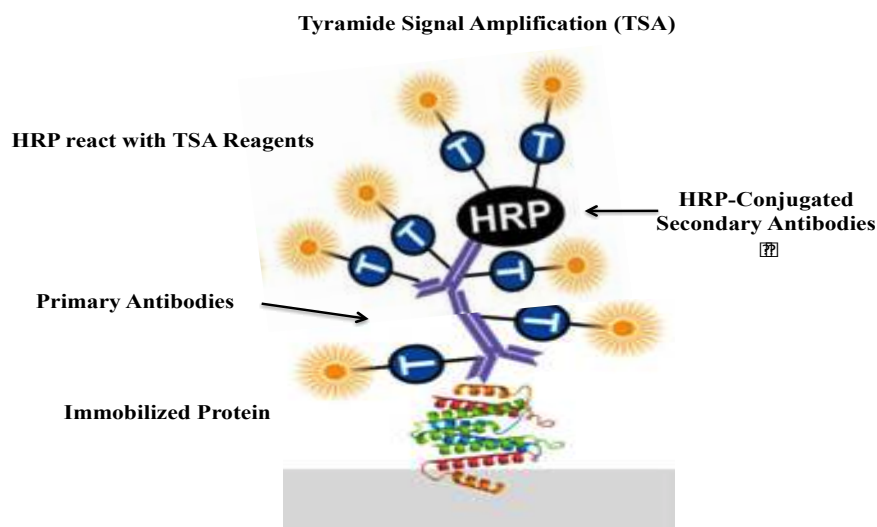


Figure 1.14: Diagram showing tyramide signal amplification (TSA).

It binds to the antibodies to produce an increased signal. HRP – horse radish peroxidase.

1.4.5. Assay validation

Assay validation can be described as 'the documentation of the performance characteristics of the method in question by specific laboratory investigations to ensure it is reliable and suitable for the analytical application'. How well the analytical data is accepted depends directly on the criteria used to validate the method. The strategy, improvement, establishment, optimisation and revalidation of the method should be covered in the validation process. Overall the validation process can be defined as a sequence of experiments that investigate the performance of an assay including the accuracy, specificity and reproducibility of a technique [166-168].

The first consideration in assay development is the intended purpose of the assay, and how to create a series of experiments that could answer the questions that could affect the assay [169]. The industrial guidelines for the production and validation of an analytical method produced by the U. S. Food and Drug Administration (US FDA), and the American Association of Pharmaceutical Scientists (AAPS) can be

modified and adjusted depending on the requirements of the analytical method being used [170].

Within the general laboratory, the major manufacturing companies (e.g. BIO-RAD and Thermo Fisher), and in house microarray systems, provide protocols for different microarray techniques that can be used. To design valid tests, the microarray process can be divided into different stages [171]. First, there is method selection: selecting the best manner to measure proteins in biological samples, and the best way to run the microarray system; second is development of the microarray system, which includes most of the optimisation steps including varying reagents and buffers to find the best combination, as this allows validation to take place and assesses the performance of the microarray; and finally there is working on the accuracy and reproducibility of the assay [172]. Optimisation and validation are a joint process whereby improving one parameter has an impact on the other, and *vice versa*.

For the purpose of this thesis, these three stages can be explained in more detail:

A) Method selection: in this thesis, the main aim was to build a methodology that was highly accurate and precise, and allows for quantifiable detection of multiple Igs simultaneously across a variety of biological samples. The microarray technique was chosen instead of ELISA as the costs were significantly lower, less sample and reagents were needed than a traditional ELISA, and the microarray had the capacity to deliver results faster than an ELISA. Microarray also has a much greater dynamic range than ELISA, with 0 to 60,000 fluorescence units compared to 0 to 4 optical density units.

B) Method development: The optimal conditions for running the microarray need to be optimised for each microarray, this includes assessment of the best printing buffer, the best blocking buffer, and the most appropriate slide surface (which was assessed by one of the students in our group). All these variables need to be inspected in depth and the optimal combination of these factors were then selected to move the microarray process forward.

C) Method performance: running a series of intra and inter variability assays over a period of days can test the performance of the assay, and can indicate the accuracy and reproducibility of the system. In this investigation the guidelines for the

parameters of the assay are based around the FDA Guidance “Bioanalytical Method Validation” [173].

Once the methodology has been validated against the equivalent ELISA, clinical samples can be applied in the microarray assay.

1.4.5.1. Parameters of assay validation

Various parameters can be inspected in the validation process for an antibody microarray platform. In this study, four parameters have been tested, specificity, precision (inter and intra-assay) and limit of detection.

1.4.5.1.1. Specificity

Cross reactivity is one of the major issues in an antibody microarray; it is possible to determine the activity of other antibodies rather than the one of interest at one time, particularly in a mixed antibody arrays. The specificity is the ability to measure the presence of an analyte when it is mixed with multiple analytes [174]. It is essential that the microarray platform can be specific for single analytes and without cross-reactivity with other analytes in the same experiment.

1.4.5.1.2. Intra assay variation

Intra assay variation can be defined as the precision acquired when the procedure is carried out under the same conditions within a short interval [175]. In the case of the microarray this is when the experiment is performed multiple times on the same slide, which acts as an indicator of how the precision is within the same day under the same conditions.

1.4.5.1.3. Inter assay variation

Inter assay variation occurs when the experiment is carried out under the same experimental conditions across a few different days. The precision is calculated and again acts as an indicator of how the effects vary in slightly different external environments (i.e. change in room temperature, light, buffers, reagents). Usually the precision is lower than that of the intra assay variation [176].

1.4.5.1.4. Limit of detection

The lower limit of detection (LOD) is the antibody concentration at which an analyte can be successfully identified. In terms of the microarray the LOD is defined as a point that is above the level of the blank; in this thesis the LOD is

calculated as the blank reading plus two times the standard deviation of the blank [177, 178].

1.5. Aims

The aim of this study was to optimise and develop a highly-advanced antibody microarray technique, applying new reagents that had never been used with this technique before. This array was then used to investigate the effects of CS on Ig class expression in the oral cavity and systemically through measuring the concentrations of different Ig types in saliva and serum specimens in healthy smoking and non-smoking subjects, and subsequently *in vitro*, in the secretions of B-cells isolated from buffy coats.

2. Optimisation of the ELISA Technique

2.1. Introduction

The enzyme-linked immunosorbent assay (ELISA), or enzyme immunoassay (EIA), is one of the most common immunological laboratory methods. This assay was first established in the 1970s as a substitute for radioimmunoassays, and is considered as one of most highly sensitive and rapid methods for measuring the concentrations of analytes. ELISAs depend on the separation of specific and non-specific substances, mainly through sequential binding of antigens and antibodies to the solid surface of a polystyrene multi-well plate. The amount of analyte correlates with the colour of the end product. Here, an ELISA was calibrated to measure immunoglobulin (Ig) isotypes and to facilitate calibration of a more advanced technique, namely a protein microarray.

2.2. Materials and methods

2.2.1. Materials

Table 2.1 shows the reagents and materials that were used in the ELISA technique, and Table 2.2 describes the types of capture antibodies that were used for our in-house ELISA.

2.2.2. Developing the ELISA technique

2.2.2.1. Choosing a suitable blocking buffer and concentration for the different immunoglobulin isotypes to generate standard curves

Capture antibodies (Table 2.2) were diluted to 1 $\mu\text{g/ml}$ (1:1000) with carbonate/bicarbonate buffer (pH = 9.2). Each well of the ELISA plates was coated with 100 μl of capture antibody and incubated overnight at room temperature. After incubation, the plates were washed 3 times with phosphate buffered saline (PBS) containing 0.05 % Tween-20. Wells of the ELISA plates were blocked either with I-block in reagent diluent (300 $\mu\text{l/well}$), 1 % bovine serum albumin (BSA; Sigma) in PBS, or with 5 % milk powder (50 mg/ml in PBS). Then, the plates were incubated for 1 h at room temperature.

Table 2.1: Reagents and materials used for the ELISA technique.

Materials	Sources
ELISA plates	Nunc plates, MaxiSorp
Carbonate/bicarbonate buffer	Sigma-Aldrich
Phosphate buffered saline (PBS)	Sigma-Aldrich
Tween-20	Sigma-Aldrich
1 % Bovine serum albumin (Sigma)	Sigma-Aldrich
I-block	Tropix, Bedford, MA, US
Biotinylated anti-human Ig Light chain κ and λ	Sigma/Oxoid
Human immunoglobulin standards	Athenas research and Technology
Human IgM, IgG, IgA and IgD ELISA Quantitation sets	Cambridge Bioscience
Streptavidin-HRP	Bio-Rad
Tetra methyl benzidine (TMB)	Thermo Scientific

Table 2.2: Concentrations of specific capture antibodies used for all Ig isotypes, as well as their sources.

Isotype	Capture Antibody	Concentration	Company
IgG	Goat anti-human IgG Fc (polyclonal)	1 μ g/ml	Sigma-Aldrich, Gillingham, UK
IgA	Goat anti-human IgA α -chain (polyclonal)	1 μ g/ml	Sigma-Aldrich, Gillingham, UK
IgD	Goat anti-human IgD (polyclonal)	1 μ g/ml	Serotec, Poole, UK
IgM	Goat anti-human IgM μ -chain (polyclonal)	1 μ g/ml	Sigma-Aldrich, Gillingham, UK

Following the incubation, the ELISA plates were washed as described above. Human immunoglobulin standards (all classes of standard Igs were obtained from Athenas Research and Technology Company) were prepared by serial 2-fold dilutions across 16 concentrations. I-block was used to dilute the purified Ig standards for wells that were blocked with I-block, while BSA was used as a diluent for purified Ig standards that were blocked with BSA or milk blocking buffers. Standard curves of IgG (100 ng/ml–0.006 ng/ml), IgA (500 ng/ml–0.030 ng/ml), IgM (1000 ng/ml–0.061 ng/ml), and IgD (500 ng/ml–0.030 ng/ml) were prepared. Next, the diluted standards were applied in triplicate to anti-Ig coated wells and incubated for 2 h at room temperature and then washed, as described above.

Subsequently, detection antibodies (a mixture of 0.5 µg/ml biotinylated anti-human Ig light chain κ and 0.5 µg/ml biotinylated anti-human Ig light chain λ antibodies were diluted in reagent diluent, added to ELISA plates, and incubated for 90 min at room temperature.

Following the incubation, the plates were washed as described above. Subsequently, streptavidin-HRP was diluted 1:200 in reagent diluent and incubated in all wells for 20 min at room temperature. After the incubation, the plate was washed as described above. Substrate reagent, tetramethyl benzidine (TMB), was applied to all wells. The plates were incubated to allow for colour development. Stop solution (1.8M sulphuric acid) was then added to stop the reaction after 5–10 min. Absorbance was measured using a FLUOstar OPTIMA Plate Reader at 450 nm (Figure 2.1).

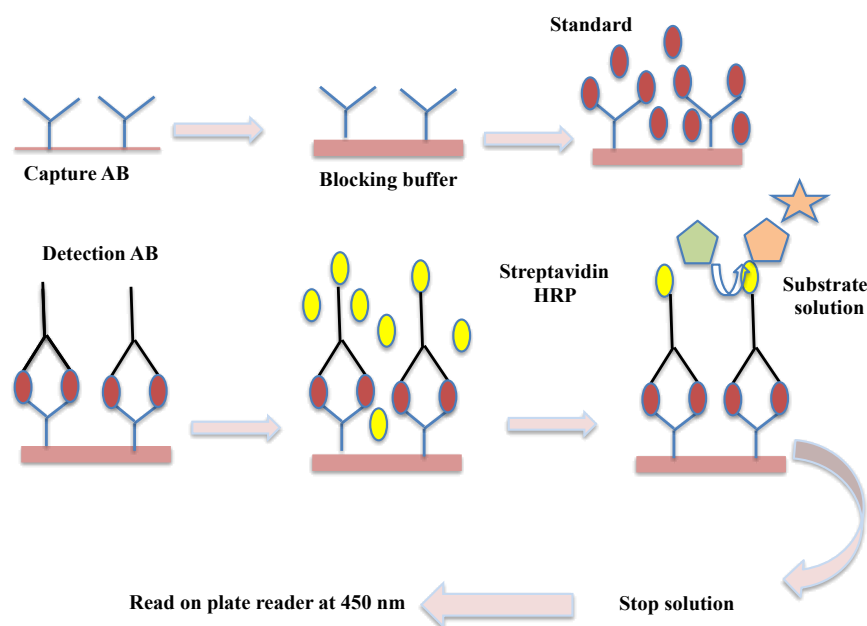


Figure 2.1: Graphical illustration of the principle of the ELISA technique.

ELISA plates are coated with capture antibodies overnight. Then, the plates are washed and blocking buffer is added. After incubation, the plates are washed again, after which standard and/or samples are added, and the plates are incubated for 2 h. Subsequently, the plates are washed and biotinylated detection antibodies are added. Then, the plates are washed and horseradish peroxidase (HRP)-conjugated streptavidin is added to the plates, which are incubated in the dark. After repeat washing, a TMB substrate is added and the reaction is stopped by adding 0.18M sulphuric acid after colour development. The absorbance is read on a plate reader at 450 nm.

2.2.2.2. Cross-reactivity experiments for studying the specificity of the in-house ELISA

Cross-reactivity experiments were performed to check the specificity of the in-house ELISA. To investigate the ELISA specificity, a specific capture antibody was incubated with a variety of immunoglobulin standards, and then an appropriate, species-matched detection antibody was added. These experiments showed whether the IgA ELISA detects only IgA (not IgG or IgM); whether the IgG ELISA detects

only IgG (not IgA or IgM); and whether the IgM ELISA detects only IgM (not IgA or IgG).

2.2.2.2.1. Cross-reactivity experiments for determining IgG specificity

Cross-reactivity experiments for in-house ELISAs were repeated 3 times for IgG capture to determine their specificity in detecting the appropriate class of antibody. In the cross-reactivity experiments, the same ELISA steps described above were followed by adding an anti-human IgG Fc-specific antibody as the capture antibody, after which serially diluted IgM, IgG, and IgA immunoglobulin standards were detected with a mixture of 0.5 µg/ml biotinylated anti-human Ig light chain κ and 0.5 µg/ml biotinylated anti-human Ig light chain λ antibodies.

2.2.2.2.2. Cross-reactivity experiments for determining IgM specificity

In this experiment, the same steps described above were followed using an anti-human IgM µ-chain-specific antibody (1 µg/ml; Sigma-Aldrich, Gillingham, UK) as the capture antibody, after which serially diluted IgM, IgG, and IgA immunoglobulin standards were detected with a mixture of 0.5 µg/ml biotinylated anti-human Ig Light chain κ and 0.5 µg/ml biotinylated anti-human Ig Light chain λ antibodies.

2.2.2.2.3. Cross-reactivity experiments for studying the specificity of the ELISA quantitation set for detecting IgM, IgG, IgA, and IgD

Human IgM, IgG, IgA, and IgD ELISA quantitation sets were obtained from Cambridge Bioscience, and cross-reactivity experiments were performed to determine their specificity in measuring only the appropriate antibody. Increasing concentrations of capture and detection antibodies were tested to determine the optimum concentrations for further experiments.

2.3. Results

2.3.1. Choosing a suitable blocking buffer and concentration for detecting different Ig isotypes to generate standard curves

The in-house ELISA was developed by testing different blocking buffers to optimise the specificity of Ig detection. BSA was found to be the best blocking

buffer for specific detection of the different Ig isotypes, as it gave the best end-product optical density values and a low background, and it generated a linear standard curve. Moreover, a carbonate-bicarbonate buffer was effective as a coating buffer for detecting all Ig isotypes because it generated a more alkaline medium than did PBS, which enhances the binding of anti-human Ig to ELISA plates. Each Ig was investigated with different concentrations to generate standard curves (Figure 2.2).

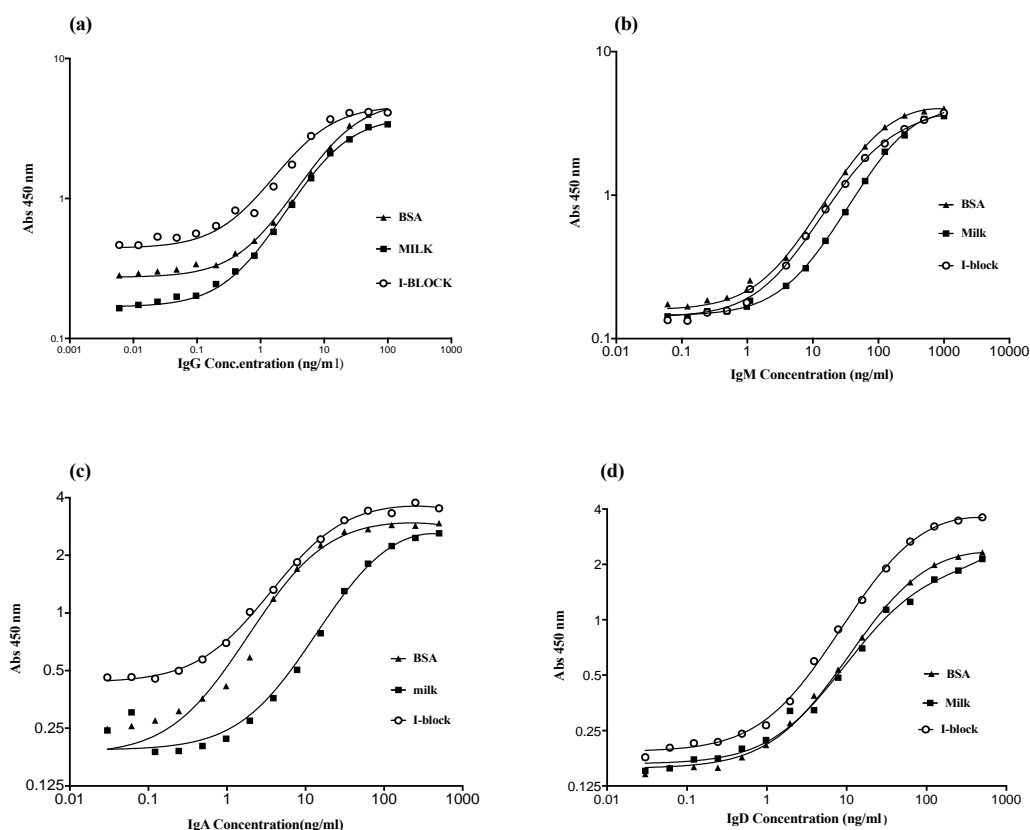


Figure 2.2: In-house developed ELISA, comparison of the results obtained using three different blocking buffers.

a) Mean ($n=3$) obtained for the IgG standard curve. b) Mean ($n=3$) obtained for the IgM standard curve. c) Mean ($n=3$) obtained for the IgA standard curve. d) Mean ($n=3$) obtained for the IgD standard curve. For each Ig type the average absorbance for the 3 different blocking buffers (3 % BSA blocking buffer, the I-Block Protein-Based Blocking Reagent, and 5 % milk powder) are shown. The I-Block reagent showed a high background and a lack of dynamic range in the standard curves, therefore BSA was chosen as a suitable blocking buffer for future experiments.

2.3.2. ELISA cross-reactivity results

2.3.2.1. Results of studying the specificity of the in-house ELISA with Igs

Cross-reactivity experiments showed that the IgG-capture antibodies detected both IgM and IgA (Figure 2.3a). In addition, the IgM-capture antibodies detected both IgG and IgA (Figure 2.3b). Based on these results, the Ig-capture antibodies were

not specific enough to yield accurate results. Thus, the Cambridge Bioscience Quantitation Set reagents were used in subsequent experiments.

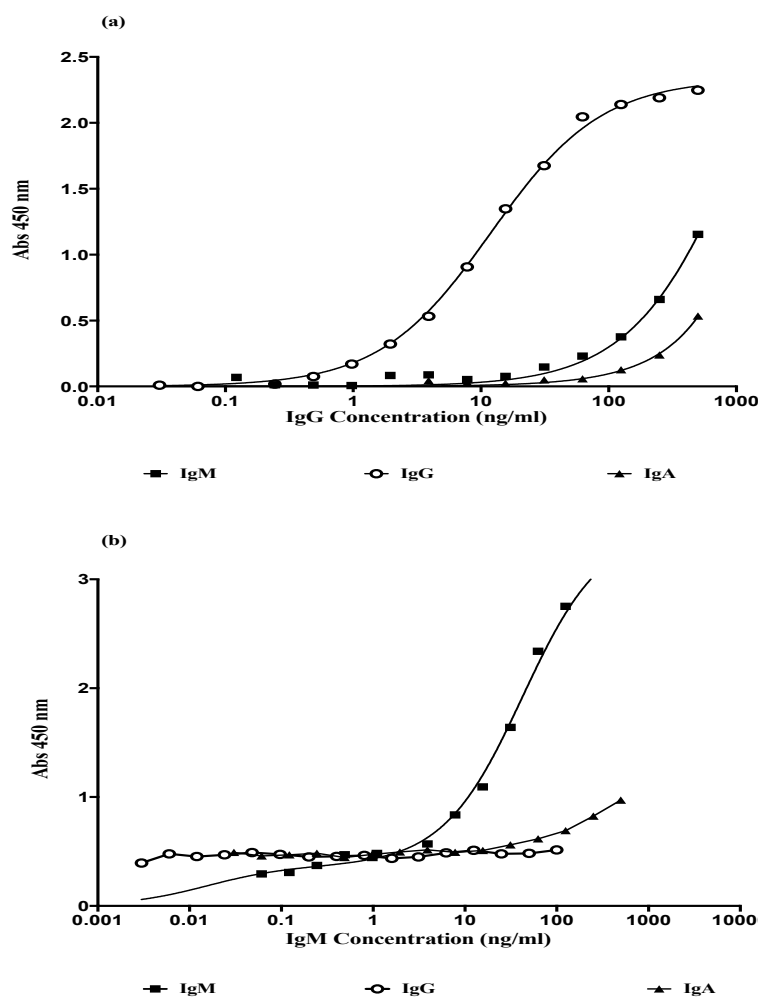


Figure 2.3: Cross-reactivity experiments for the in-house ELISA.

(a) The IgG ELISA showed high cross-reactivity with IgM and IgA, with higher values detected for IgM than IgA when the IgG-specific capture antibodies were used. (b) The IgM ELISA showed high cross-reactivity with IgG and IgA, with higher values for IgA than IgG when the IgM-specific capture antibodies were used, but this could also be interpreted as a high background in the case of IgG.

2.3.2.2. Specificity of the ELISA Quantitation Set in detecting IgM, IgG, IgA, and IgD

Results from cross-reactivity experiments demonstrated the high specificity of the ELISA Quantitation Set. As shown in Figure 2.4, the IgG-capture and secondary antibodies measured only IgG (not IgM or IgA). Similarly, IgM ELISA detected

IgM, but not IgG or IgA; the IgA ELISA detected IgA, but not IgM or IgG; and the IgD ELISA only detected IgD, using this set of reagents.

Due to the many advantages of advanced microarray techniques, the same reagents were used to optimize the performance of the microarray-based test.

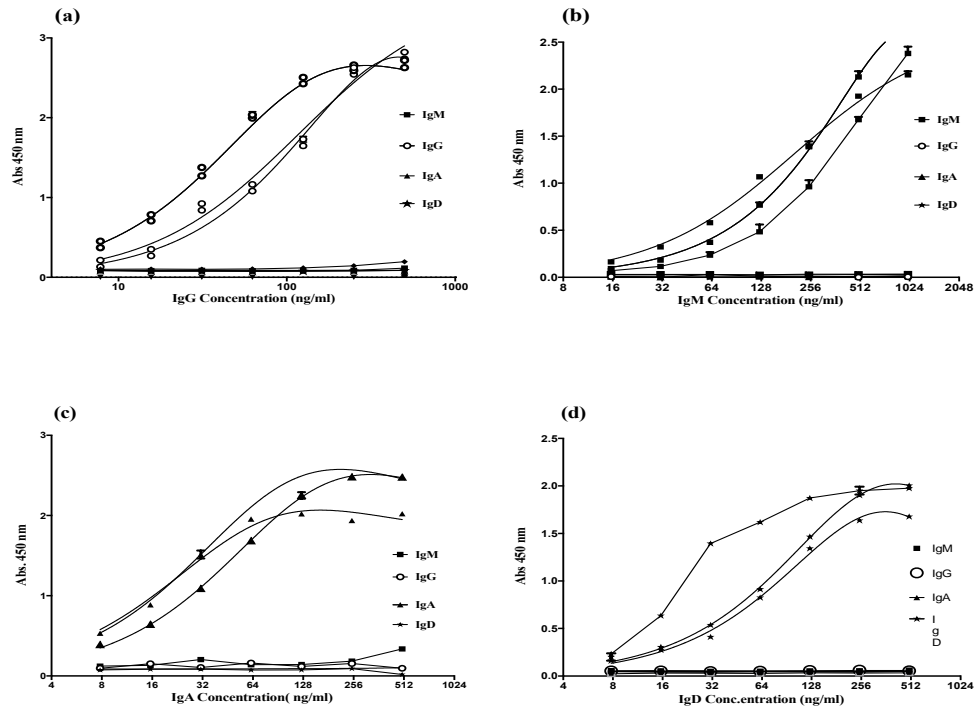


Figure 2.4: Investigation of cross-reactivity between anti-Ig capture and detection antibodies of different Ig class specificities with Ig standards of different classes.

(a) IgG standard curves with high specificity; the capture and detection antibodies measured only IgG, but not IgM, IgA, or IgD. (b) IgM standard curves with high specificity; the capture and detection antibodies measured only IgM, but not IgG, IgA, or IgD. (c) IgA standard curves with high specificity; the capture and detection antibodies measured only IgA, but not IgG, IgM, or IgD. (d) IgD standard curves with high specificity; the capture and detection antibodies measured only IgD, but not IgG, IgM, or IgA. The IgD standard was not curve-fitted; thus, the data points shown were connected with joining lines.

2.4. Discussion

In the experiments described in this chapter, the “sandwich” ELISA technique was optimised and specific Ig isotypes were sandwiched between anti-heavy chain antibodies and secondary, labelled anti-light chain antibodies in ELISA plates. During coating, a polyclonal anti-human heavy chain Ig isotype (mouse IgG) antibody was used as a capture antibody to bind Igs. Carbonate-bicarbonate buffer (pH 9.2) was selected as a coating buffer, and it was found that 1 µg/ml of anti-Ig antibodies produced detectable levels of Ig isotypes. In addition, the specificity and sensitivity of the assays were evaluated. Assay specificity was investigated by determining whether the IgA, IgG, and IgM ELISAs detected only the intended Ig class, or if cross-reactivity was also observed. When evaluating the assay sensitivity, it was important to ensure that the Ig concentrations in samples were determined at the linear ranges of the standard curves. The in-house generated ELISA showed high cross-reactivity between Igs, which may have been compounded by the use of anti-heavy and -light chains secondary antibodies; these antibodies can bind to all types of Igs and produce a detectable signal in cases where the capture antibody is not pure or specific enough to detect only a single isotype. To reach accurate conclusions, experiments were performed with reagents from Cambridge Bioscience, which showed sufficiently high specificity and sensitivity to assess antibody levels in serum and saliva samples. In these experiments, capture and secondary antibodies recognizing the heavy chain of the targeted Ig isotype were used to ensure specificity of the test.

2.5. Conclusions

The reagents from Cambridge Bioscience gave high specificity and accuracy in the measurement of Ig isotypes, and due to the advantages of the microarray techniques in evaluating the levels of analytes, the next chapter focuses on experiments designed to select appropriate reagents for this technique, which were subsequently used to measure the concentrations of antibodies in samples.

3. Optimisation and Validation of Antibody Microarray

3.1. Introduction

The antibody microarray technique is used to measure the quantity of proteins that are found in biological samples such as serum, sputum, and urine. The principle of this technique is based on the use of specific capture antibodies printed onto the surface of microarray slides, which can bind their cognate proteins in a sample. After the initial interaction of the capture antibody with the target protein, a secondary antibody is added to detect the capture antibody-target protein complex. In general, microarrays are based on a principle that is similar to that of enzyme-linked immunosorbent assays (ELISAs), but microarrays often involve reduced costs and hands-on time. In addition, microarrays can be used to analyse a single slide several times, which enables validation of the results.

ELISAs are commonly used as a gold-standard technique because they offer high sensitivity and specificity; however, a substantial body of research has been conducted using microarrays to determine serum antibody concentrations. Antibody microarrays are considered to represent an emerging, useful approach for detecting disease biomarkers; thus, optimisation and validation studies should be performed to facilitate application of this technology in medical research [179, 180].

Three different types of protein microarrays have been developed. Reverse-phase protein microarrays offer specificity in detecting modifications of intracellular proteins in cell lysates that accompany certain diseases. Functional protein microarrays enable the determination of interactions between proteins and other significant molecules. Finally, analytical protein microarrays can be used to study protein affinities and protein concentrations, as was done in the current research [156, 181].

During the analytical protein sandwich microarray process, different antibodies were printed on microarray slides to serve as capturing antibodies for the analytes of interest. Then, the slides were probed with samples, followed by direct detection or incubation with secondary analyte-specific biotinylated antibodies, that were identified with a fluorescent dye (sandwich microarray) [182]. The principle of sandwich antibody microarrays is demonstrated in Figure 3.1. Basically, capture

antibodies are printed onto a slide, after which a blocking buffer is added to block non-specific binding to the slide. Next, the samples are added, followed by the addition of secondary biotinylated antibodies and, finally, a streptavidin-conjugated fluorescent dye.

In general, numerous factors can affect the data generated by an antibody microarray, including the types of capture antibody and samples used, the slide composition (plastic or glass), and the slide coating (silicon or polylysine), which can affect the affinity of each slide for substrates. Selecting an appropriate surface chemistry helps generate high-intensity signals after applying unknown samples. In addition, protein adsorption and covalent bonding can affect the antibodies that attach to the slide surface. These types of slides are recognized as 2-D surfaces, whereas slides coated with a polymer matrix are recognized as 3-D surfaces. Physical adsorption occurs through hydrogen bonding, ionic bonding, and/or hydrophobic interactions, which immobilize the antibodies onto the slide surface. Weak, non-covalent interactions can result in partial antibody binding; however, this is generally preferable to covalent binding, which can change the protein structure. Moreover, the choice between direct detection or detection with secondary, biotinylated antibodies, as well as the blocking and printing buffers used can affect the signal intensity and the background level.

Different types of printing buffers can be used; thus, it is important to select one that does not alter the biological characteristics of the printed proteins. PBS-trehalose has been used as a printing buffer and contains a naturally occurring sugar (trehalose), which has a strong ability to protect proteins from dehydration and denaturation, thereby helping to maintain the natural structures of printed proteins [183]. Another important buffer that should be considered is the blocking buffer. The blocking buffer should be capable of blocking all the other active sites on the slide surface to prevent unfavourable binding to other proteins, except for the capture antibodies [179]. In this study, glass slides coated with poly-L-lysine were printed with highly specific capture antibodies, which led to capture-antibody immobilization by adsorption. Furthermore, BSA was used as a blocking buffer because it resulted in a low background, compared to other buffers tested.

In brief, the main advantages of microarrays over ELISAs are as follows; microarrays require a lower amount of sample to run the assay, with significantly

reduced time and expense. Microarrays also provide the ability to perform multiple replicate measurements with the same antibodies during the same experiment, under typical experimental conditions. Furthermore, microarrays can be used to identify several different proteins over a range of concentrations, in the same experiment. The multiplexing capabilities of microarray also enable 4-6 replicates to be performed in a single assay (compared with the 2-3 normally used in ELISA) and the post assay analysis procedures then allow poor-performing replicates to be excluded based on valid criteria (e.g. spot circularity, uniform pixel density, etc.), thereby reducing the coefficient of variation for sample replicates.

3.2. Materials and methods

All reagents were warmed to room temperature (20–25 °C) before use. These reagents included coating buffer (0.05 M carbonate/bicarbonate buffer, pH 9.6), wash solution (0.50 mM Tris, 0.14 M NaCl, 0.05 % Tween 20, pH 8.0), blocking solution (50 mM Tris, 0.14 M NaCl, 1 % BSA, pH 8.0), and sample/conjugate diluent (50 mM Tris, 0.14 M NaCl, 1 % BSA, 0.05 % Tween-20). Table 3.1 shows all materials that were used in the microarray technique.

3.2.1. Materials

Table 3.1: List of materials used for the microarray technique.

Materials	Sources
Biotinylated anti-human IgG (H&L)	Biolegend
BSA	Sigma-Aldrich
Carbonate/bicarbonate buffer	Sigma-Aldrich
DMSO (dimethyl sulfoxide)	Sigma, UK
Goat anti-human IgM,	Cambridge Bioscience
Goat anti-human IgA	Cambridge Bioscience
Goat anti-human IgG	Cambridge Bioscience
HRP conjugated Goat anti-human IgG-Fc	Cambridge Bioscience
I- block	Tropix, Bedford, MA, US
NaCl	Fisher
PBS-Trehalose	Sigma-Aldrich
Phosphate buffered saline (PBS)	Sigma/Oxoid
Poly-lysine-slides	Sigma-Aldrich
streptavidin Cy5 fluorochrome	Bio-Rad
Teramide amplification	Bio-Rad
Tris-phosphate	Calbiochem
Tween-20	Sigma-Aldrich

3.2.2. Printing slides on the arrayer

The Human IgM, IgG, IgA, and IgD Quantitation sets from Cambridge Bioscience contained all essential reagents required for the experiments discussed in this chapter. Each kit included the necessary capture antibody, a standard, and a detection antibody. All reagents were reconstituted for the optimisation experiments. Initially, the capture antibodies were prepared at a concentration of 100 µg/ml by dilution in printing buffer (1 × PBS containing 50 mM Trehalose; Sigma-Aldrich). Then, 20 µl of printing buffer was added to each well of the microarray plate, and the capture antibodies were printed on poly-L-lysine slides (Catalogue No. 63478-AS; Electron Microscopy Sciences, USA). The plate was covered with an aluminium cover and centrifuged for 3 min at 1200 × *g* at room temperature. Finally, the plate was placed in the biobank in a MicroGrid II arrayer. The slides were placed on a tray in the arrayer, and the required program was run to print 16 blocks. For each block, we set the program to print a specific antibody 5 times in separate rows, and this process was repeated twice in the same block to obtain a total of 10 spots for each antibody, but in two separate sets. The printed slides were preserved inside a vacuum once the printing was completed. Further details of the printing process and the number of slides that could be printed from a given volume of reagent are given in section 1.4.2.

3.2.3. Amplification

One issue seen was low signal intensity with some of the proteins at low concentrations. To overcome this problem, an extra amplification step can be performed with protein microarrays to increase the signal intensity, as demonstrated in previous studies [161, 184, 185]. In the current research, tyramide amplification was used for this purpose. Tyramide amplification is based on the use of an HRP-conjugated detection antibody in the presence of H₂O₂, which triggers the activation and oxidation of biotinylated tyramide, which binds covalently to proteins at electron-rich amino acid residues. This results in increasing the amount of biotin present at immuno-reactive sites, causing an improvement in the sensitivity and signal intensity. In addition, the background level can potentially increase with amplification, which may be because the emission wavelength of Cy5 changes from 450 nm (without tyramide amplification) to 380–400 nm (with amplification).

3.2.4. Slide preparation

Capture antibody (goat anti-human IgG-Fc) was diluted to 100 µg/ml in 2 different printing buffers (carbonate/bicarbonate buffer and PBS-trehalose) to compare them. Two wells of the microarray plate were coated with 20 µl capture antibody, and the plate was covered with an aluminium cover and centrifuged at 1,200 x g. Then, the plate and polylysine slides were placed in a block in the arrayer, and a programme was run to print 16 blocks in each slide. Slides were held under a vacuum overnight. On the next day, the slides were blocked with I-block in reagent diluent (100 µl/block) or BSA blocking buffer to compare these blocking buffers. Then, the slides were shaken for 1 h at room temperature. After incubation, the slides were washed 3 times with PBS containing 0.05 % Tween-20 on the shaker for 3 min each wash. During incubation, human IgG standard was prepared at a concentration of 500 ng/ml and serially diluted with reagent diluent with 2-fold dilutions across 8 points. The resulting concentrations in the dilution series were 1000, 500, 250, 125, 62.5, 31.25, and 15.6 ng/ml. One block was used as a negative control, into which only the reagent diluent was added.

Following incubation, the slides were washed as described above. Then, 100 µl of the HRP-conjugated detection antibodies were added and the slides were incubated for 1 h at room temperature on a shaker.

Following incubation, the plates were washed, as described above. Subsequently, 100 µl tyramide amplification reagent was prepared (4:1:4) applied to the slides, and incubated for 10 min at room temperature. After the incubation, the slides were washed as described above, and 100 µl Cy5-conjugated streptavidin (1:1,000 in 3 % BSA) was applied to each block. The slides were incubated for 15 min on the shaker in the dark. Finally, the slides were released from the holder, washed in ultra-pure water for 5 min, and centrifuged for 3 min. Fluorescence was detected using a GenePix scanner 4200AL at a wavelength of 635 nm (Figure 3.1).

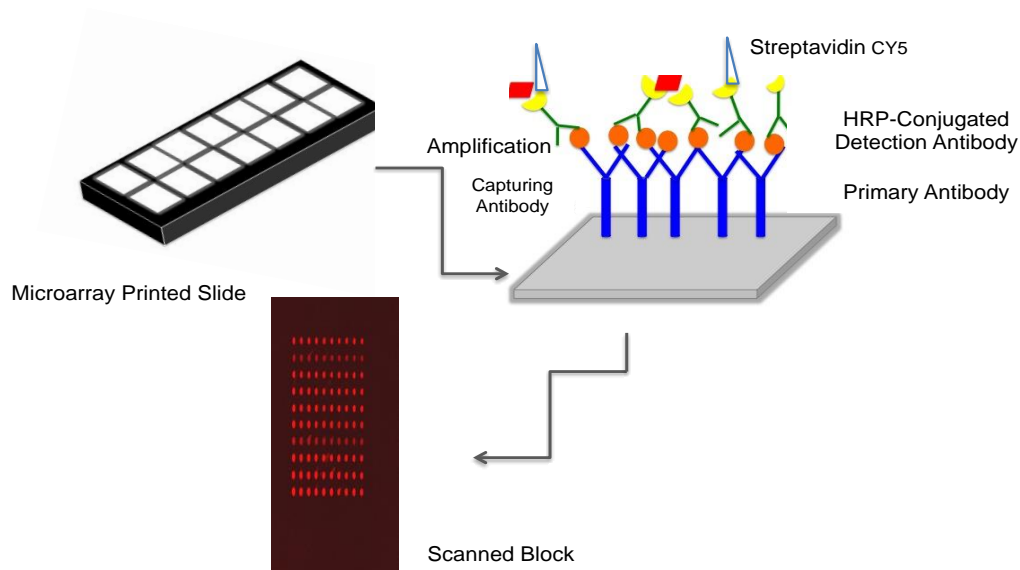


Figure 3.1: Principle of the sandwich antibody microarray.

Initially, a specific capture antibody is printed on poly-L-lysine-coated slides. Subsequently, serum or saliva samples or Ig standard are applied to the slide and attach to the immobilized capture antibodies on the slide. After a 1-h incubation period, an HRP-conjugated secondary antibody is added, then biotinylated-tyramide amplification reagent was added and Cy5-conjugated streptavidin was used to detect bound antibodies by scanning the slides in a laser-based scanner. The signal intensities produced at each spot were proportional to the antibody concentration in the sample.

3.2.5 Scanning of slides

After processing, slides were immediately scanned with a GenePix 4200AL microarray scanner, to measure the fluorescent dye (Cy5 Fluor 635). The GenePix scanner has a dynamic detection of signal above background noise in a range of 0 - 65,535; therefore, setting the laser power was a crucial step to obtain the lowest background noise and highest signal intensities. After investigation, these adjustments were made to prevent photo bleaching: 80-90 % laser power, (photo-multiplier tube voltage set to PMT 370-400, 10 μm pixel size, 20 μm focus position, and a scanning wavelength of 635 nm (laser standard red). Slides were inserted in to the slide carrier, loaded into the scanner, scanned, and images produced were saved in the manufacturer's recommended format (multiple-image TIFF).

3.2.6 Slide analysis

After scanning the slides, a GenePix Array List (GAL) file was analysed using Axon GenePix Pro 6 Microarray Image Analysis software (version 6, USA) to detect the spots that were scanned. This file was first created in Excel, using the same order of the capture antibodies in the plate, as follows:

Plate	Row	Column	Name	Dilution
1	A	1	IgG capture antibody	1
1	B	1	IgM capture antibody	1
1	C	1	IgA capture antibody	1
1	D	1	IgD capture antibody	1

Then, a file was saved as a comma-defined file (csv), which was then converted to a GAL file by following the steps in the Clone Tracking Wizard in the computer of the arrayer. In GenePix Pro, when ready for the analysis, the GAL file was associated with the spots to be classified. The signal intensities of the scanned spots were converted in GenePix Pro 6 to numerical values and gpr files were generated containing multi-parametric data, including the raw data for each arrayed protein, localization, identification variables, signal intensity and background noise. The median fluorescence of each spot was measured and the background values were subtracted. The GenePix software read spots according to their morphologies, allowing all spots with irregular shapes or low signals to be filtered (i.e. spot circularity < 65) and excluded using Microsoft Excel 2010; the data were further processed using GraphPad Prism 6.0 software (GraphPad Software, USA) (Figure 3.2, Figure 3.3).

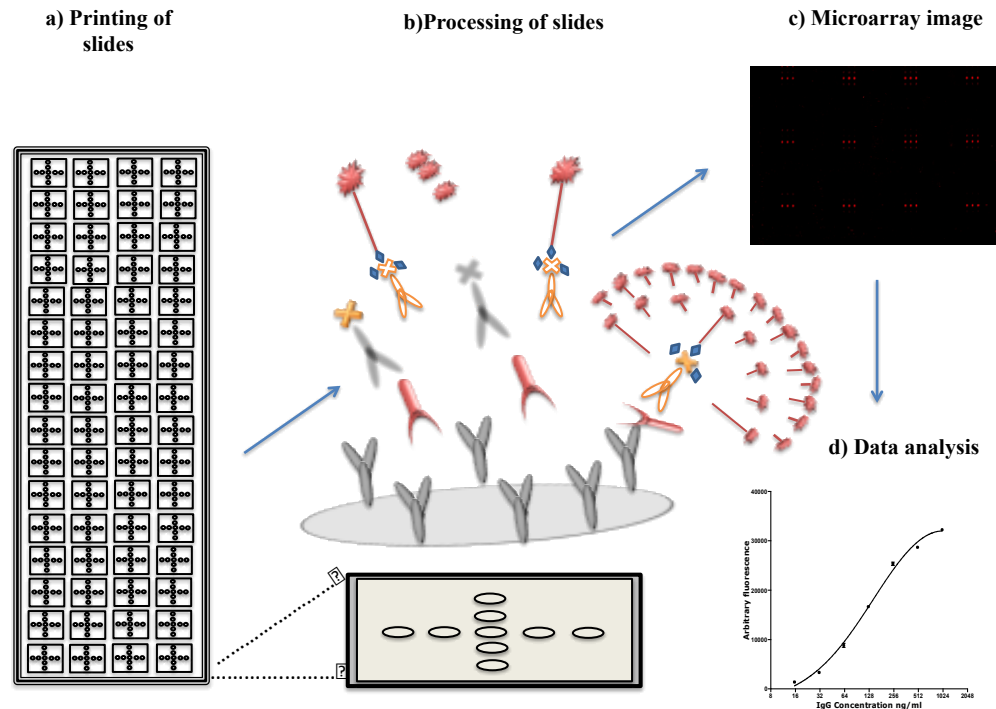


Figure 3.2: Diagram of the microarray assay protocol for quantification of total Ig antibodies.

(a) printing: a printed slide of 64-pads, containing printing replicates across each pad, (b) capture anti-human Igs antibodies used to bind total Igs in serum/saliva, subsequently detected with secondary antibodies, (c) fluorescent image of a pad, and (d) data analysis of the scanned images.

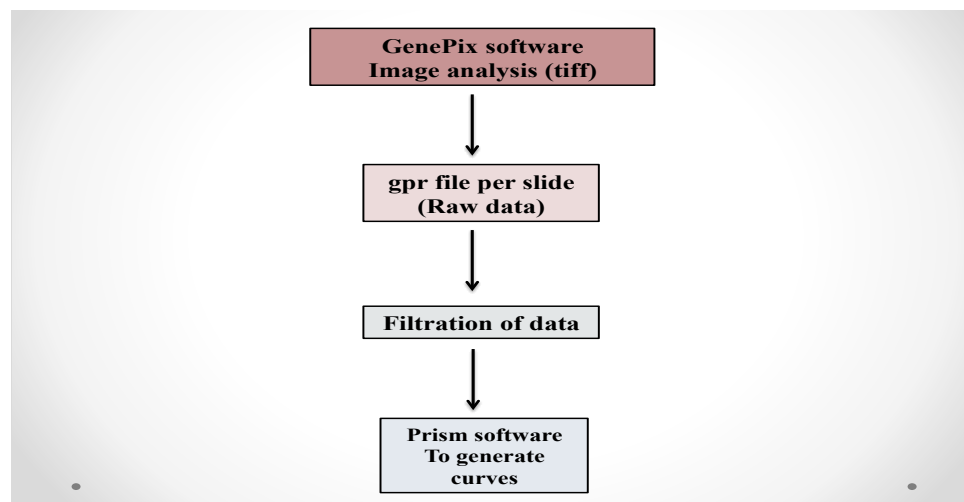


Figure 3.3: Schematic diagram shows the steps for generating the numerical numbers and curves.

3.3. Developing the antibody microarray

The purpose of these experiments was to develop suitable Ig standard curves that could be used to measure Igs in serum and saliva samples.

3.3.1. Generating an IgG standard curve

3.3.1.1 Determining the best printing buffer

The capture antibody (goat anti-human IgG-Fc) was diluted to 100 µg/ml in 2 different printing buffers (carbonate/bicarbonate buffer and PBS-trehalose) to compare them. Two wells of the microarray plate were coated with 20 µL. Then, the assay was completed, following the steps described above. HRP-conjugated goat anti-human IgG-Fc was used as secondary antibody and biotinylated tyramide amplification reagent was applied, followed by streptavidin-Cy5 and DMSO was used to fix the spots.

3.3.1.2 Determining the optimal blocking buffer

In this experiment, slides were coated with capture antibody (goat anti-human IgG-Fc) in trehalose printing buffer containing either I-block or BSA (BSA with NaCl and Tris-phosphate) in each of 8 blocks. The highest concentration of IgG was 125 ng/ml, which was serially diluted with reagent diluent (2-fold dilutions) across 8 points. Secondary HRP-conjugated goat anti-human IgG-Fc antibody was applied, followed by biotinylated tyramide amplification reagent and then streptavidin-conjugated Cy5.

3.3.1.3 Using a biotinylated secondary antibody

To improve the signal intensity, the secondary antibody was changed from a non-biotinylated to a biotinylated detection antibody. In this experiment, 2 dilution series were prepared; in the first series, the standards ranged from 125 ng/ml to 1.95 ng/ml, while the second dilution series started at a concentration of 1.95 ng/ml. Biotinylated anti-human IgG (H & L) was used as a detection antibody, instead of the HRP-conjugated goat anti-human IgG-Fc, and therefore the tyramide amplification reagent was not applied. In addition, the capture antibody was applied at 50 and 100 µg/ml to determine if there was any difference between these concentrations. However, this secondary antibody showed a lack of specificity, and

it was therefore decided it would be better to use the HRP-conjugated, goat anti-human, IgG-Fc with amplification instead.

3.3.1.4 Using an amplification reagent and an HRP-conjugated goat anti-human IgG-Fc as a secondary antibody at different concentrations

In this experiment, 5 different concentrations of the non-biotinylated, HRP-conjugated, goat anti-human IgG-Fc secondary antibody were prepared for use in IgG standard curves (1:100,000, 1:200,000, 1: 400,000, 1:800,000 and 1:1,600,000). The human IgG standard was prepared at a high concentration of 500 ng/ml, which was serially diluted with reagent diluent by 2-fold increments across 8 points.

3.3.2. Generating IgM and IgA standard curves

The previous procedure was applied to perform the assay. Capture antibody (goat anti-human IgM, goat anti-human IgA, and goat anti-human IgG) were diluted to 100 µg/ml in PBS-trehalose buffer. Subsequently, 2 wells of the microarray plate were coated with 20 µl of these capture antibodies. The human IgM and IgA standards were prepared at the top concentration 500 ng/ml, then diluted with reagent diluent by 2-fold steps across 8 points. Three secondary anti-body concentrations of HRP conjugated Goat anti-human IgM and Goat anti-human IgA were prepared (1:400,000; 1:800,000 and 1:1,600,000).

3.4. Antibody microarray validation test

3.4.1. Cross-reactivity test

Cross-reactivity assessment is an important validation parameter in protein binding-based assays, and in the case of this study, antibody microarray platforms. To determine if cross-reactivity occurred in this microarray platform, the following experiments were conducted: first, to examine the specificity of detecting standard isotypes, a microarray panel was printed with specific capture antibodies, different protein standards were added, and then a particular detection antibody was used. Secondly, to examine the specificity of the capture antibody, a microarray panel was printed with different capture antibodies, after which various standards were

added, followed by the addition of detection antibodies and downstream processing (Figure 3.4).

3.4.1.1. Examination of the cross-reactivity for IgG with IgM and IgA by using biotinylated anti-IgG (H & L) as detection antibody

Based on the previous result, which gave rise to a good standard curve with biotinylated anti-human IgG (H & L), the cross-reactivity was checked.

3.4.1.2. Examination of IgG cross-reactivity with IgM and IgA using an HRP-conjugated, goat anti-human IgG-Fc as the detection antibody

Based on the previous result, the cross-reactivity was checked to determine the specificity of the reagents. HRP conjugated Goat anti-human IgG-Fc (1:800,000) was used as a secondary antibody.

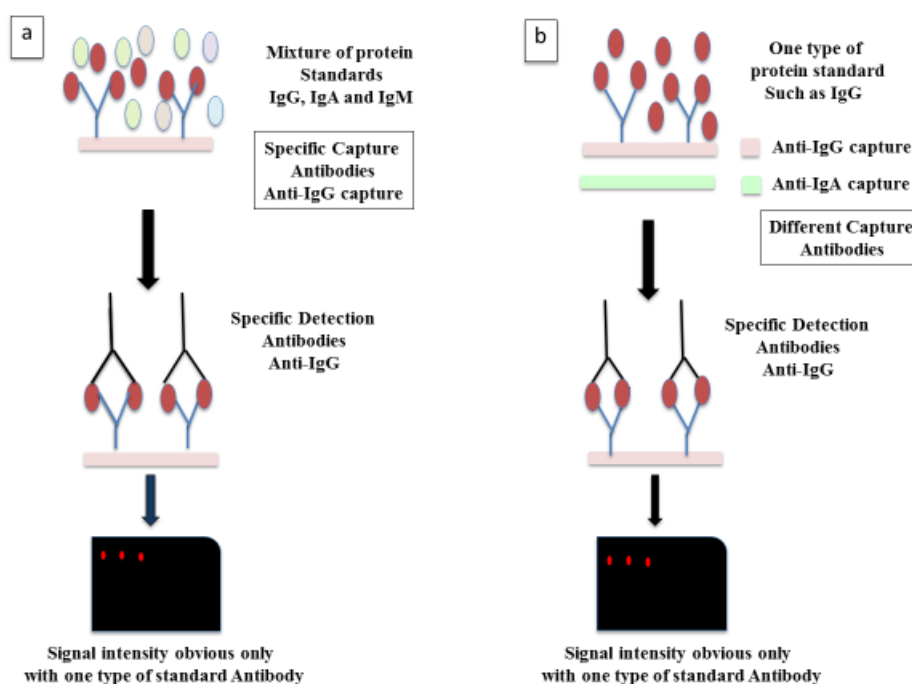


Figure 3.4: Determining the specificity of capture and detection antibodies.

a) A mixture of Ig standards was applied to the same pad; only one class of Ig was able to bind to the capture and detection antibodies (exemplified here detection of IgG). **b)** Different capture antibodies were printed on different pads (exemplified here with anti-IgG or anti-IgA), but only the specific one has the ability to bind to the corresponding Ig standard (exemplified here with IgG).

3.4.1.3. Examination of the cross-reactivity for IgM with IgG and IgA, also IgA with IgG and IgM

Four different wells of the microarray plate were coated in order, with 20 µl of capture antibody (Goat anti-human IgG-Fc, IgM-Fc, IgA-Fc, and IgD-Fc), which was diluted to 100 µg/ml in a PBS-Trehalose printing buffer. Human IgG, IgM, IgA and IgD standards were prepared at the top concentration 125, 500, 500, and 500 ng/ml, respectively, and diluted with a reagent diluent by 2-fold steps across 8 points. Then the same procedure was applied to complete the assay. HRP conjugated goat anti-human IgM (1:800,000) and IgA (1:1,600,000) were prepared and applied to the slide.

3.4.2. Precision tests

3.4.2.1. Intra- and inter-assay variability

Intra-assays and inter-assays were performed to establish the precision of the microarray assay. In the case of intra-assays, the experiments were repeated on the same slide on the same day while, in inter-assays, experiments were performed on three different days. Up to 6 serum samples from healthy donors were collected and diluted for measuring immunoglobulins. Then, the mean and standard deviation of the replicates were calculated to estimate the precision of the microarray assay.

3.5. Results

3.5.1. Developing the antibody-microarray

3.5.1.1. Determining the best printing buffer and blocking buffer

Previous studies in our group showed that PBS-trehalose printing buffer yielded better spot shapes than did the carbonate/bicarbonate buffer [231], making it suitable for printing slides in the arrayer. BSA showed less background than did I-block, so BSA was selected as a blocking buffer for the microarray studies (Figure 3.5).

3.5.1.2. Determining the best capture-antibody concentration for printing onto slides and using a biotinylated secondary antibody

To determine the effect of the capture antibody concentration, a comparison between two capture-antibody concentrations (50 and 100 ng/ml) was conducted

(Figure 3.6). The results showed that 100 ng capture antibody/ml generated better standard curves than the 50 ng/ml did. In addition, the results showed a good standard curve was produced when the higher concentration of 125 ng/ml was used, while the dilution series that was generated with 1.95 ng/ml as the highest concentration did not generate any spots (Figure 3.7). However, the biotinylated secondary antibody showed poor specificity (section 3.5.2.1.1); therefore, the secondary antibody was changed to the HRP-conjugated goat anti-human IgG-Fc.

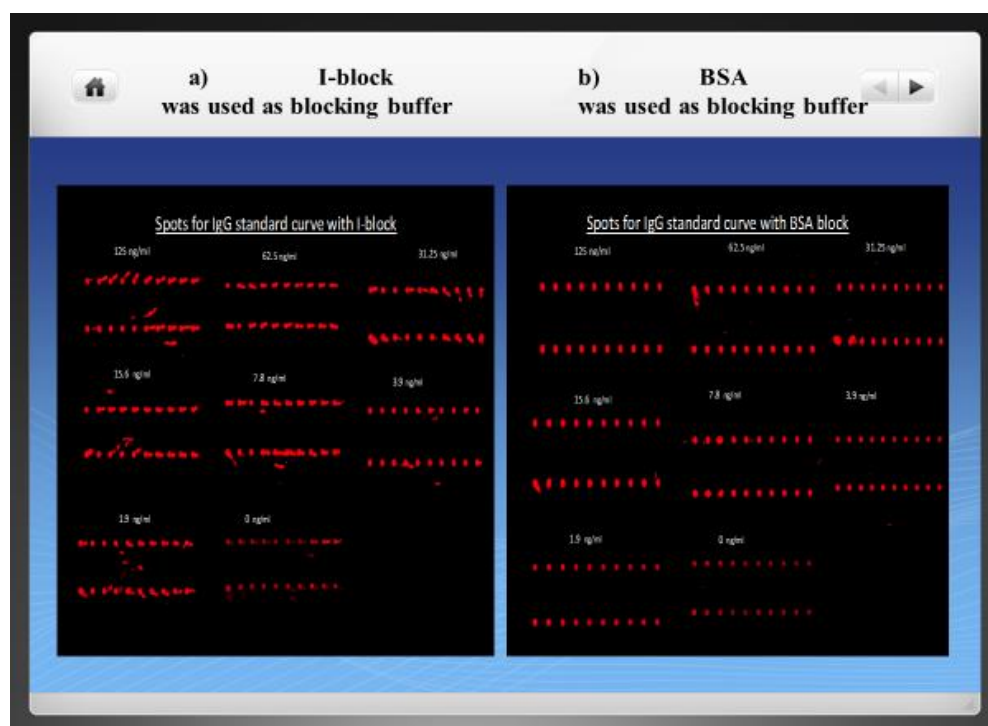


Figure 3.5: Determining blocking buffer suitability.

Poly-L-lysine slides were printed and processed with two different blocking buffers (BSA and I-Block), BSA (b) improved the level of signal intensity in terms of the shapes of the spots, compared to I-Block (a).

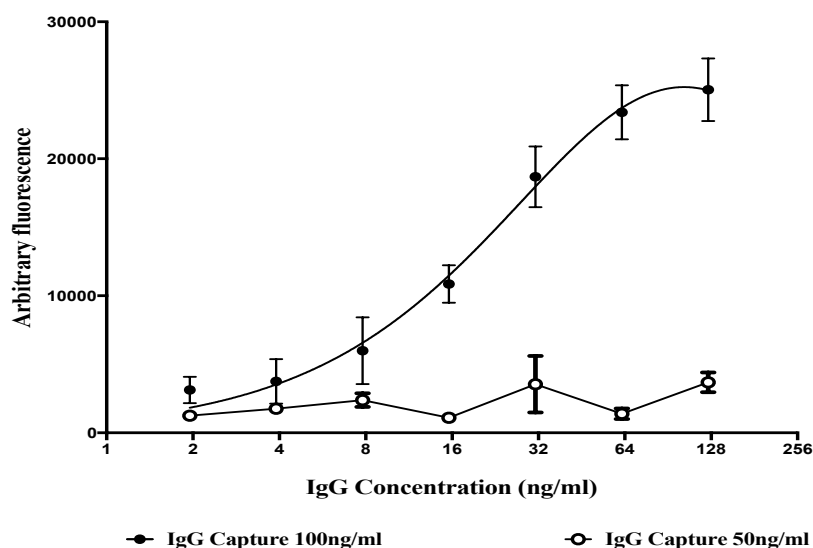


Figure 3.6: Determining the capture antibody concentration.

A substantial drop in signal intensity was observed when the capture antibody was printed at a concentration of 50 ng/ml compared to 100 ng/ml. Although this is the result of a single experiment, it suggests that 100 ng/ml anti-human IgG-Fc was the optimal capture antibody concentration for printing antibodies on poly-L-lysine slides.

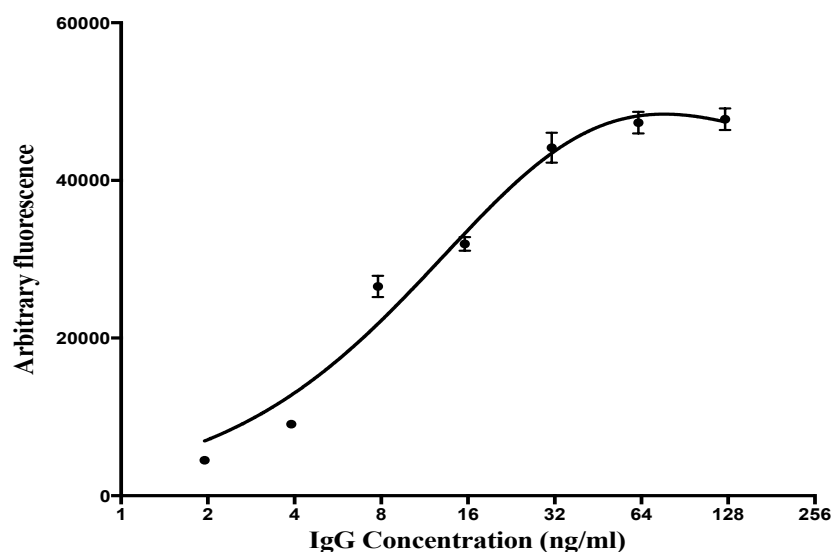


Figure 3.7: Different dilution of IgG used for standard curves.

The highest two concentrations tested in the dilution series were 125 ng/ml and 1.95 ng/ml, which were diluted 8 times to produce a standard curve. Using a top concentration of 125 ng/ml with a biotinylated anti-human IgG (H & L) as the secondary antibody improved the linearity of the IgG standard curve, while using a top concentration 1.95 ng/ml did not generate a linear standard curve.

3.5.1.3. Using an amplification reagent and HRP-conjugated, goat anti-human IgG-Fc as a secondary antibody at different concentrations

The HRP-conjugated, goat anti-human IgG-Fc secondary antibody was diluted to 1:100,000, 1:200,000, 1:400,000, 1:800,000, and 1:1,600,000. The results (Figure 3.8, 3.9) showed the 1:400,000 dilution produced saturated spots, whereas the 1:100,000 and 1:200,000 dilutions showed flat curves, therefore a secondary antibody dilution of 1:800,000 was chosen for the IgG standard curve.

3.5.1.4. IgM and IgA standard curves

The results (Figure 3.10) showed that when using a high concentration of 500 ng/ml, the HRP-conjugated goat anti-human IgM-Fc and anti-IgA-Fc were suitable as secondary antibodies, and linear standard curves for IgM and IgA were achieved with dilutions 1:800,000 or 1:1,600,000, respectively.

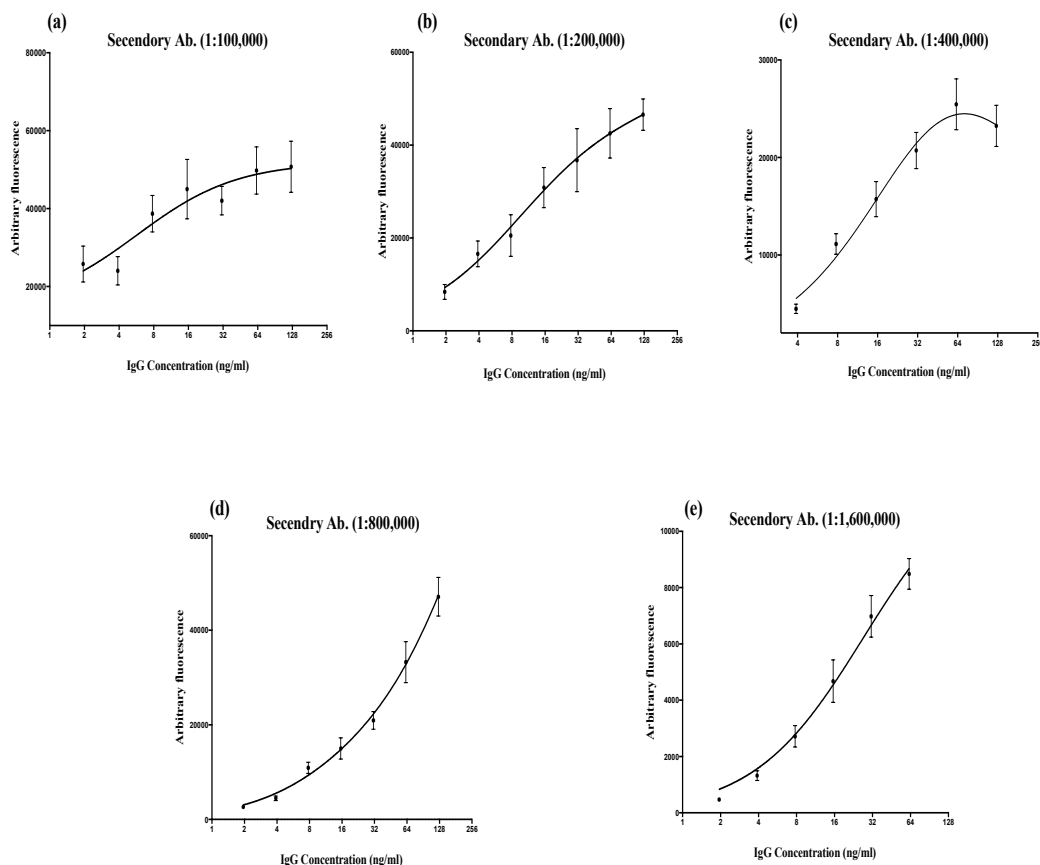


Figure 3.8: Examining different concentrations of an HRP-conjugated, goat anti-human IgG-Fc secondary antibody to improve the IgG standard curve.

The results were obtained by using four different detection-antibody concentrations. (a, b) The 1:100,000 and 1:200,000 dilutions showed flat curves and did not show gradually increasing signal intensity. (c) The 1:400,000 dilution was marked with saturated spots on the top. (d, e) The 1:800,000 and 1:1,600,000 dilutions showed good, incremental standard curves.

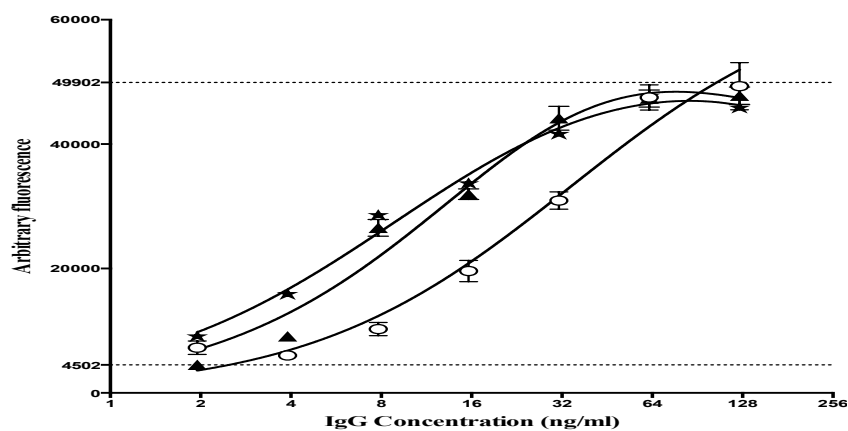


Figure 3.9: IgG standard curves generated on three different days. A secondary antibody dilution of 1:800,000 was chosen for the IgG standard curve.

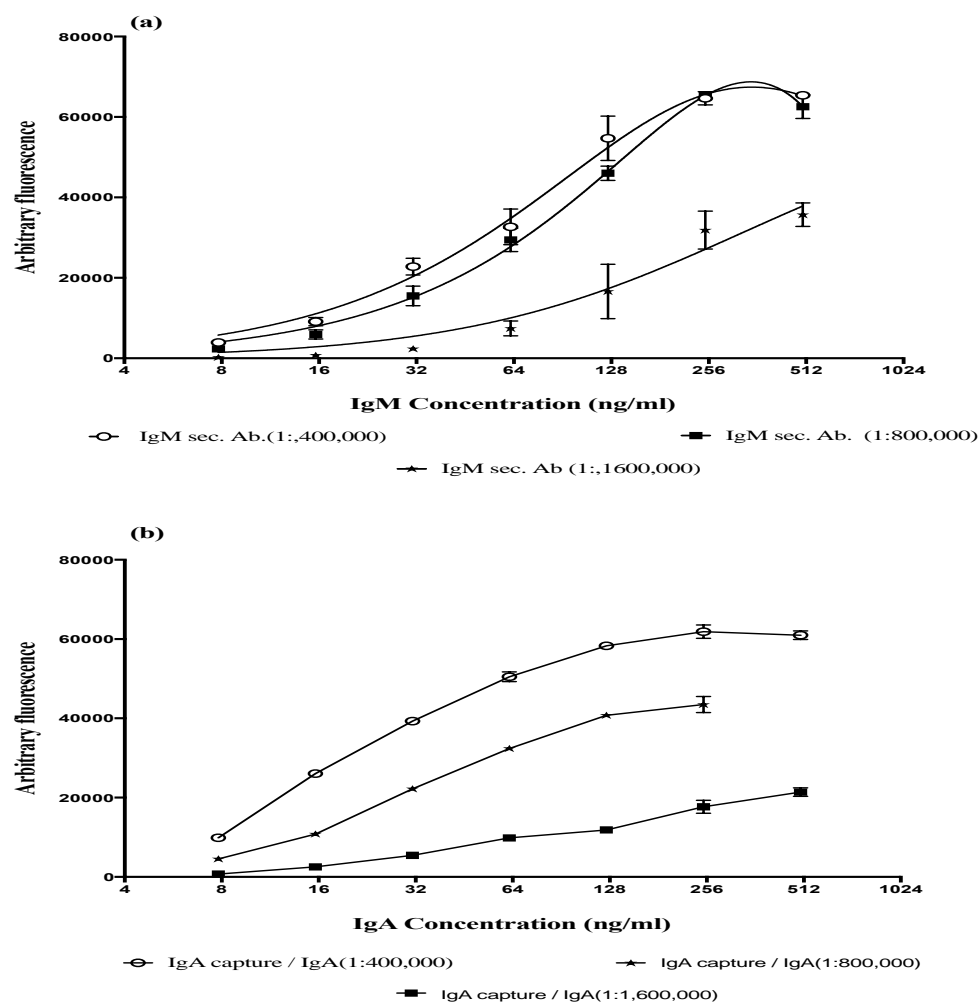


Figure 3.10: Examination of the different concentrations of the HRP-conjugated goat anti-human IgM and anti-IgA.

HRP-conjugated goat anti-human IgM-Fc secondary antibody was used to generate the IgM standard curve (a), and the HRP-conjugated goat anti-human IgA-Fc secondary antibody was used to generate the IgA standard curve (b). Dilutions of 1:800,000 and 1:1,600,000 were chosen for the IgM and IgA standard curves, respectively.

3.5.2. Antibody microarray-validation results

3.5.2.1. Cross-reactivity results

3.5.2.1.1. Examination of anti-IgG cross-reactivity with IgM and IgA, using biotinylated anti-IgG (H & L) as a detection antibody

Based on the previous results, which yielded linear standard curves with biotinylated anti-human IgG (H & L), the cross-reactivity was checked. The results showed high cross-reactivity with IgM and IgA (Figure 3.11).

3.5.2.1.2. Examination of the cross-reactivity for anti-IgG with IgM and IgA when using HRP-conjugated goat anti-human IgG-Fc as a detection antibody

Based on the previous results, cross-reactivity was checked to determine the specificity of the reagents. An HRP-conjugated goat anti-human IgG-Fc antibody (1:800,000) was used as the secondary antibody.

The results (Figure 3.12 a,b) showed a very good standard curve for IgG without any cross-reactivity with IgM and IgA, also, IgD was not detectable. Moreover, there was no false-positive detection between the secondary antibody and IgM. This result indicated the high specificity of the secondary antibody, as well as the IgM and IgA capture antibodies. To ensure reproducibility this experiment was performed on 3 different days and the results were averaged.

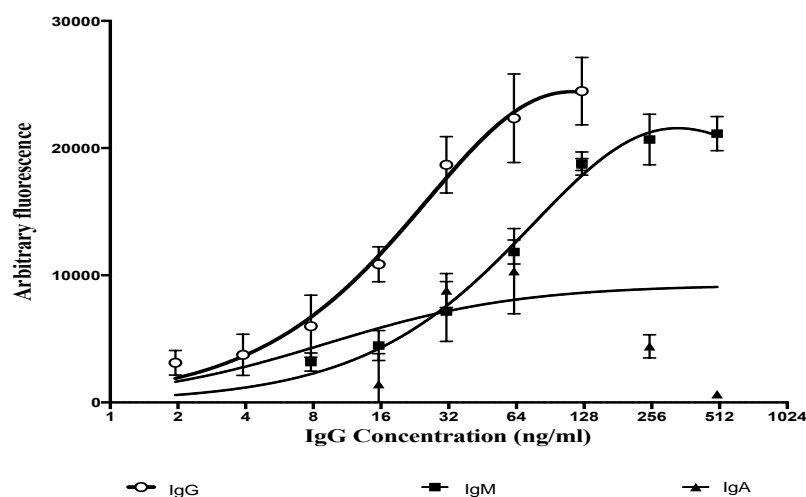


Figure 3.11: Cross-reactivity experiments.

The biotinylated anti-human IgG (H & L) antibody used showed high cross-reactivity with IgM and IgA. The irregular distribution of points for IgA is the reason for poor fit of the curve.

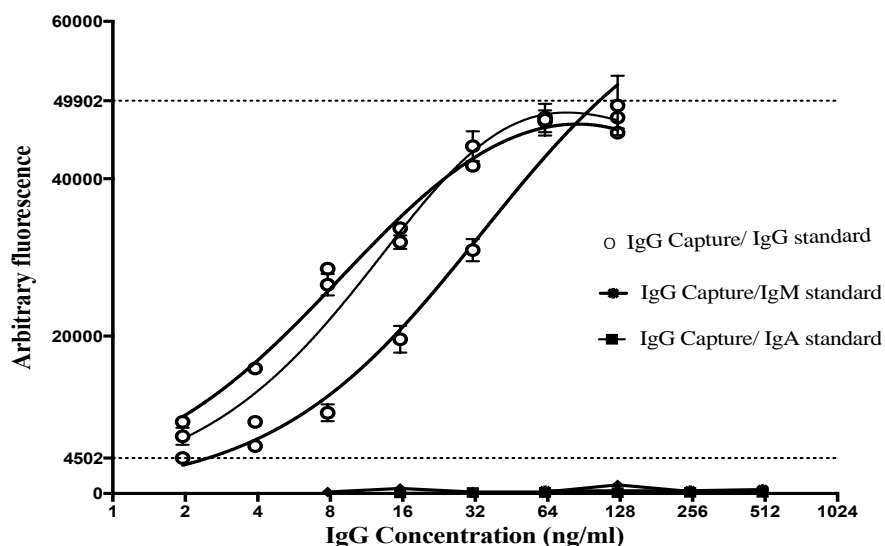


Figure 3.12 (a): Studying cross-reactivity between IgM, IgA and IgD standards with anti-IgG.

Anti-human IgG was used as the capture antibody, and an HRP-conjugated goat anti-human IgG-Fc antibody served as the detection antibody. No cross-reactivity was observed between the IgM and IgA or IgD standards with an anti-human IgG capture antibody and an HRP-conjugated goat anti-human IgG antibody as the detection antibody. IgG standard curves were generated on three different days. The values for IgD were completely undetectable and therefore do not appear on this figure.

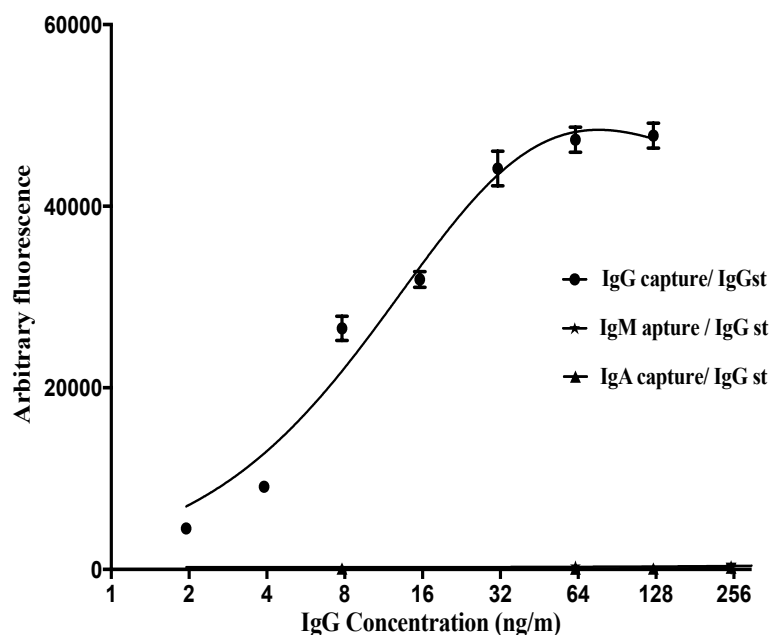


Figure 3.12 (b): Studying potential binding between IgG and different capture antibodies.

Different capture antibodies were coated on the slide and incubated with an IgG standard and an HRP-conjugated goat anti-human IgG-Fc antibody as the detection antibody. The data shown in this figure confirmed that the IgG standard could bind only with the IgG capture antibody, but not with the IgM, IgA or IgD capture antibodies. The values with IgD capture antibody were completely undetectable and therefore do not appear on this figure.

3.5.2.1.3. Examination of the cross-reactivity for anti-IgM with IgG and IgA, as well as that for anti-IgA with IgG and IgM

The results showed that no cross-reactivity occurred between IgM or IgG with the anti-IgA and that the IgA capture and detection antibodies measured only IgA (Figure 3.13 a, b). Moreover, no spots developed when applying IgA and IgG standards with the anti-IgM antibody and its secondary antibody (Figure 3.14 a,b). Additional experiments were performed that included secondary IgG and IgM antibodies, or IgA with a secondary anti-IgM antibody. Analysing the slides showed that IgG only bound with its capture and detection antibodies. The same results were also found with IgA (Figure 3.15), and also, IgD was not detectable.

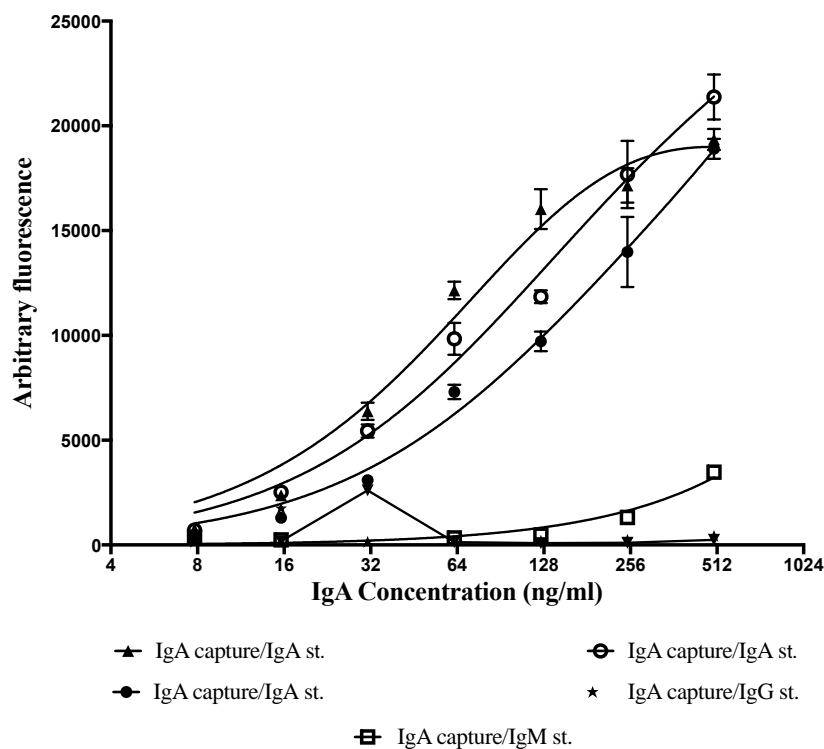


Figure 3.13 (a): Studying the cross-reactivity between IgG, IgM standards and IgD with anti-IgA antibodies.

Using anti-human IgA as a capture antibody, an HRP-conjugated goat anti-human IgA as a detection antibody, and different Ig isotypes. The data shown confirmed the specificity of the IgA capture and secondary antibodies because the IgM and IgG standards were not detected significantly. IgA curves were done on three different days. Also, IgD was not detectable and so does not appear in the figure.

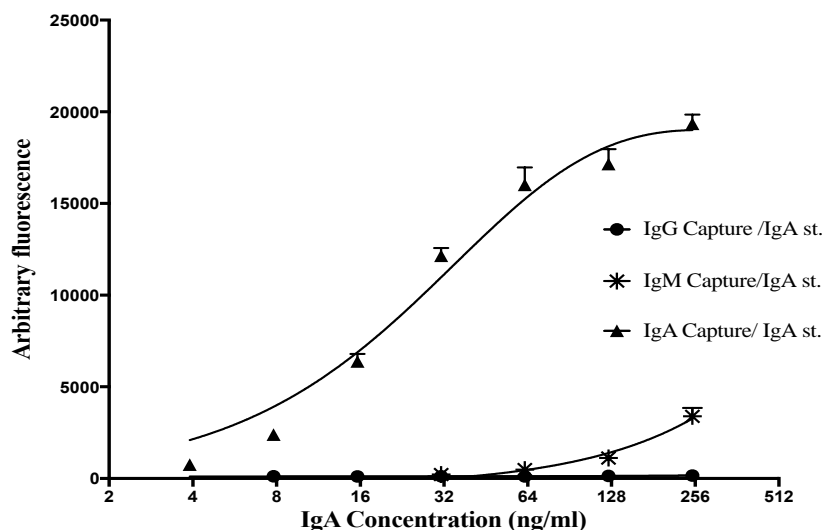


Figure 3.13 (b): Studying potential binding between IgA and different captures antibodies.

The slide was coated with different capture antibodies, incubated with IgA standard, and binding was detected with an HRP-conjugated goat anti-human IgA. This experiment confirmed that the IgA standard could bind only with the IgA capture antibody, but not significantly with the IgM or IgG capture antibodies.

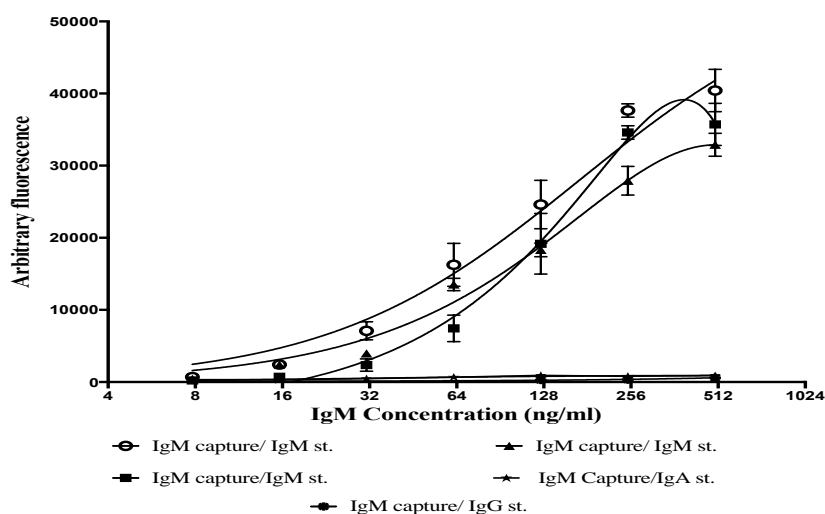


Figure 3.14 (a): Studying the cross-reactivity between IgG and IgA standards with anti-IgM antibodies.

Using an anti-human IgM as the capture antibody, an HRP-conjugated goat anti-human IgM as the detection antibody, and different Ig isotypes. The data shown confirmed the specificity of the IgM capture and secondary antibodies because the IgA and IgG standards were not detected. The curves for IgM standard represent three different experiments.

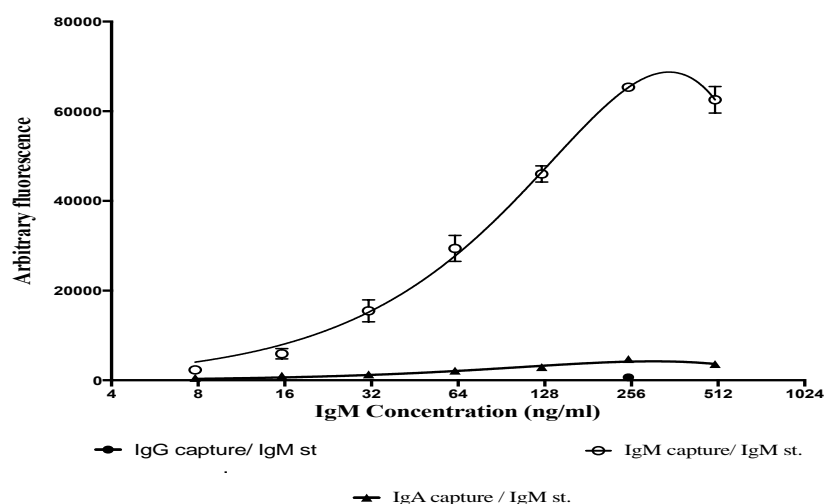


Figure 3.14 (b): Studying potential binding between IgM and different captures antibodies.

The slide was coated with different captures antibodies, incubated with primary IgM, and detected with an HRP-conjugated goat anti-human IgM. The data shown in this figure confirmed that the IgM standard could bind only with the IgM capture antibody and not to the IgG or IgA capture antibodies.

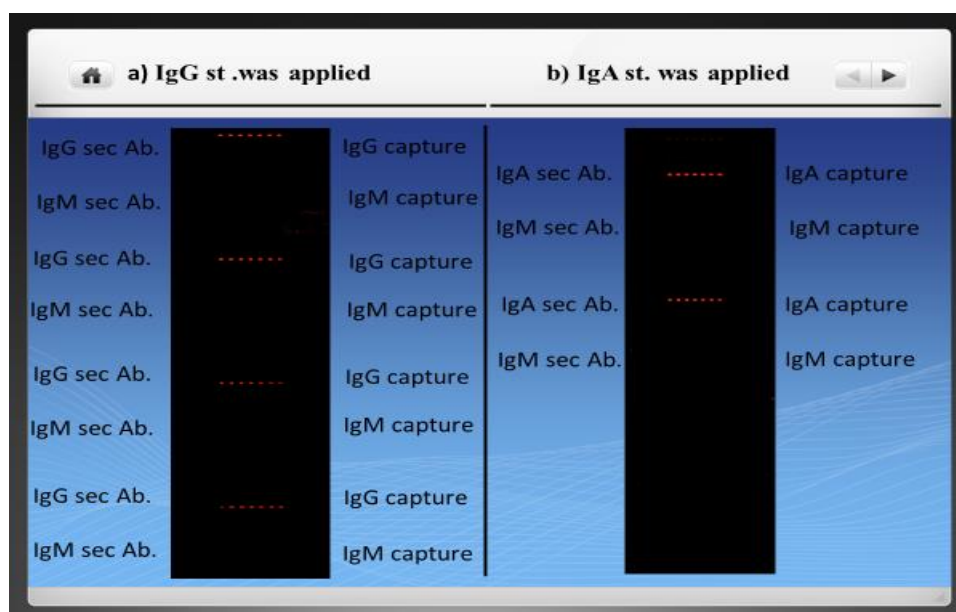


Figure 3.15: Example of slides used for studying the cross-reactivity of IgG and IgA standards with the IgM capture and detection antibodies.

Every block showed high specificity with the reagents used, as the IgG spots were observed only with the IgG capture and detection antibodies, and no spots were observed with the IgM capture and detection antibodies. Similar results were obtained with IgA.

3.5.2.2. Precision test results

3.5.2.2.1. Intra- and inter-assay variability results

The coefficients of variation (CV) for the intra-assay tests were calculated for several serum samples, and the readings varied by less than 10% for IgG, IgM, IgA, and IgD, which was acceptable for studying samples from smokers and non-smokers in the microarray assay (Figure 3.16). As expected, the inter-assay CVs were greater than the intra-assay CVs, and the readings were less than 15 % for IgG, IgM, IgA, and for 7 IgD samples (Figure 3.17). These CVs can be accepted according to FDA [255].

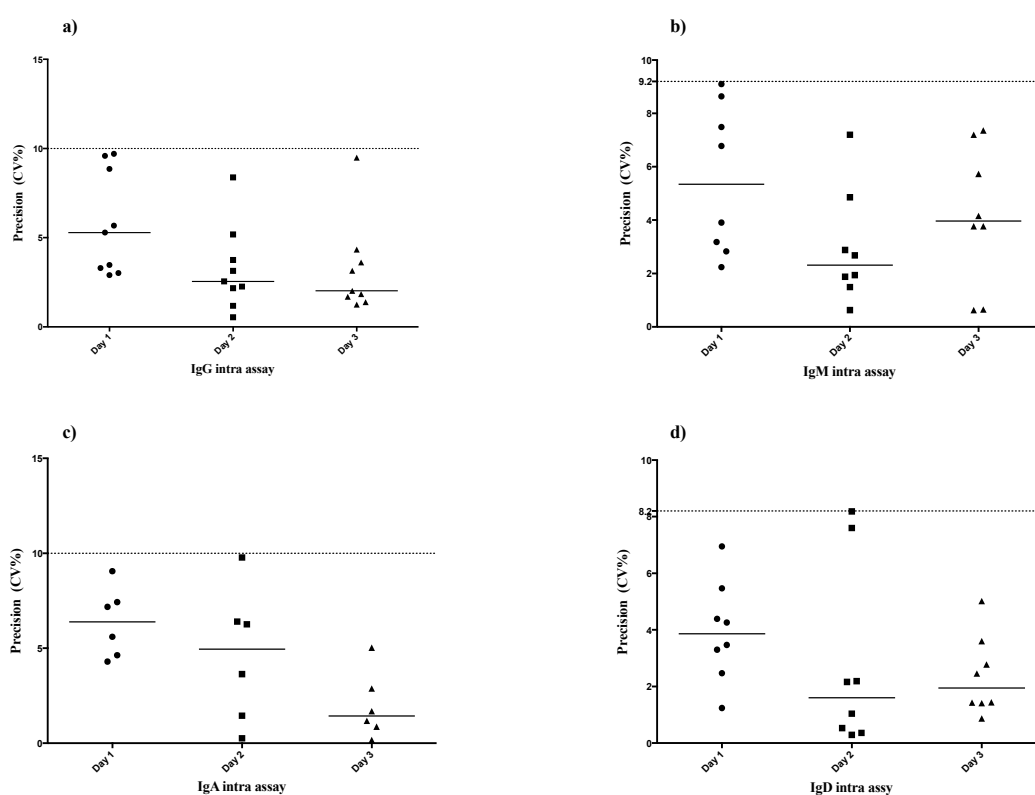


Figure 3.16: The coefficients of variation (CVs) of intra-assay results were calculated for several samples.

The dashed lines represent the acceptable limit of precision ($CV \leq 10\%$). The solid lines represent the median for each group. The CV% was $<10\%$ for IgG (a), $<9.2\%$ for IgM (b), $<10\%$ for IgA (c), and $<8.2\%$ for IgD (d).

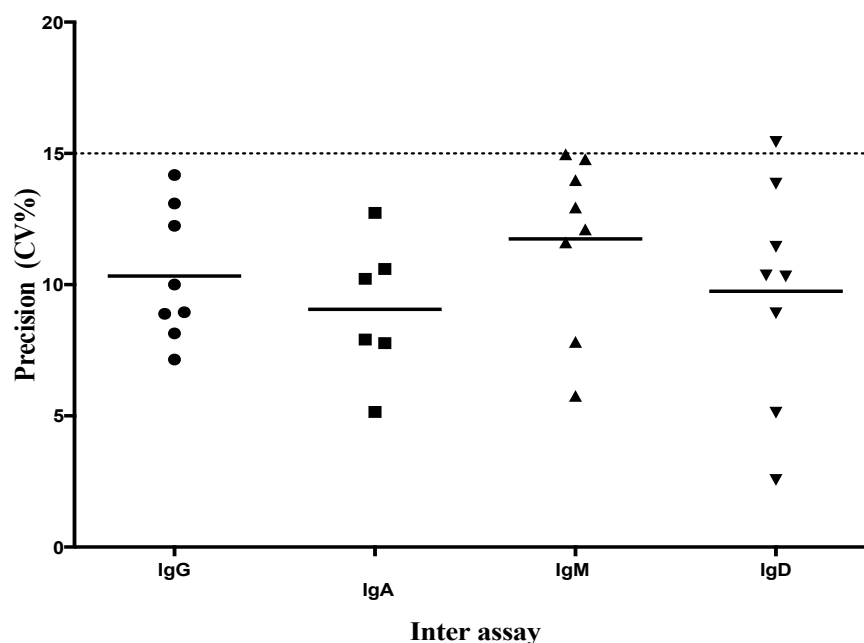


Figure 3.17: The percent coefficient of variation (CV %) for inter-assay test results was calculated for several serum samples.

The solid lines represent the median for each group. The dashed lines represent the acceptable limit of precision ($CV \leq 15\%$) for IgG, IgM, IgA, and IgD.

3.5.2.2.2. Limit of detection (LOD)

The LOD can be defined as the lowest concentration of analyte that can be reliably detected. The LOD was determined for each antibody by calculating the mean and standard deviation of the blank from the standard curve, after which the following equation was applied: $LOD = 2SD (\text{mean of the blank}) + \text{mean of the blank}$ (Figure 3.18). The LOD for IgG was 1685; whereas the LOD for IgM (–28), IgA (7), and IgD (2447) were all below the lower limit in the standard curves.

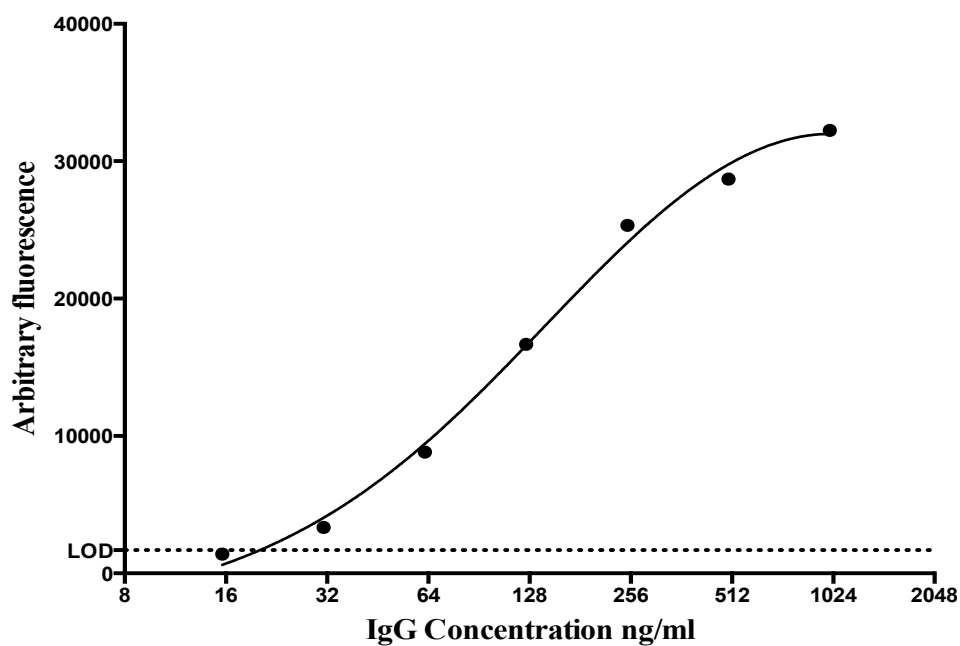


Figure 3.18: Determining the LLOD for IgG.

The lower limit of detection (LLOD) for IgG was determined as lowest level of analyte that could be measured using the standard curve. $LOD = 2SD$ (mean of the blank) + mean of the blank.

3.6. Discussion

In this chapter, all essential parameters that can affect microarray sensitivity and reproducibility were evaluated and optimized. Within this study, the parameters optimized included the printing buffer, blocking buffer, and the concentrations of the standard and detection antibodies. A commercial ELISA kit was optimised to make it suitable for detecting the Ig classes, using a microarray platform.

Poly-L-lysine slides were chosen for this study based on previous data from our group. Another important factor that affects development of an antibody microarray is non-specific protein binding, which increases the background and spot signals. Therefore, optimization of the blocking buffers I-Block and BSA (with tris-phosphate and NaCl) was carried out to reduce non-specific binding. By comparing the effects of different blocking buffers on the sensitivity and specificity of Ig detection, BSA (with tris phosphate and NaCl) was shown to produce the highest-quality performance when compared to other blocking buffers tested, generating regularly shaped spots with a low background, and lacking a tail. Moreover, PBS-trehalose buffer was effective as a coating buffer for the detection of all the Ig isotypes, as it involves a more alkaline medium that enhances anti-human Ig binding to microarray slides. Each Ig was investigated using different concentrations to generate optimal standard curves, and standard curves for all Igs were successfully generated in the microarrays.

Intensive cross-reactivity experiments were performed in this chapter to identify cross-reactivity with the detection antibodies. The optimal concentration of the standard antibodies was found to be 500 ng/ml; however, the concentration still showed a high level of cross-reactivity when using biotinylated anti-IgG (H & L) as the detection antibody. Therefore, additional experiments were performed to eliminate cross-reactivity in our antibody microarray platform. These experiments involved investigating the effect of using an *HRP-conjugated goat anti-human IgG-Fc*. And interestingly, the results showed that no cross-reactivity occurred, which could have been due to the ability of all antibodies to recognize light chains when use biotinylated anti-IgG (H & L), while the HRP-conjugated detection recognized part of heavy chain which is highly specific for each isotype. Precision was also examined in this chapter by assessing the intra- and inter-assay CV %. The results showed that the intra-assay variation for all antibody classes was below 10 %,

which was acceptable, and the intra-assay on different days was acceptable at <15 %.

3.7. Conclusions

The use of microarray technology is currently increasing. This chapter details the optimization and validation of an antibody microarray using very specific and accurate reagents, which are able to detect IgG, IgM, IgA, and IgD classes in large numbers of samples. As shown in the next chapter, the antibody microarray was used to test 100 samples, but this could be expanded to include more samples and in a high throughput manner. The results of this chapter demonstrated that the developed microarray could identify the isotype of multiple samples relatively easily, with a smaller sample volume than required for the equivalent standard ELISA assay and with a greater dynamic range of detection. As both the accuracy and reproducibility of the microarrays were acceptable, serum and saliva samples were analysed in microarrays to study differences between samples from smokers and non-smokers, as described in the next chapter.

4. Comparing Antibody-Microarray Data from Smokers and Non-Smokers

4.1. Introduction

The chronic inhalation of cigarette smoke changes immunological functions by impacting both innate and adaptive immunity. The incidence of many diseases could be associated with the adverse effects of cigarette smoke on the immune system, which may cause inflammatory responses and affect the tissues and bodily organs. Therefore, a comparison of smokers' and non-smokers' immunoglobulin levels can provide critical insights into the mechanisms of smoking-related diseases. Although the effects of cigarette smoking on humoral and cellular immunity have been investigated, variable results have been reported in these studies, and additional studies should be performed [123, 186]. The microarray technique has been used in the present study to provide novel insights into the pathogenesis of smoking-related diseases.

4.2. Materials and methods

4.2.1. Procedure for serum and saliva collection

Serum and saliva were collected from 48 smoking (median age = 30 years; age range 19-51) and 48 non-smoking (median age = 32.5 years; age range 20-56) male blood bank volunteers at King Abdulaziz Hospital, Jeddah, Saudi Arabia. The samples were aliquoted in eppendorf tubes and stored at -20°C. They were transported on ice to Queen's Medical Centre, Nottingham, UK, and then stored frozen until assayed. Ethical approval was granted by The Biomedical Research Ethics Committee, Ministry of Higher Education, King Abdulaziz University, Faculty of Medicine (a copy of the approval letter is appended).

4.2.1.1. Collection of demographic data on the subjects

Tables 4 and 5 show demographic information for the male smoker subjects. Non-smoking male subjects were age-matched with the smoking subjects, who had an average age of 30 years – there are no significant differences in the age between the two groups. The demographic information on age, years smoked and number of

cigarettes smoked per day were self-reported by each subject and recorded on a standardised form. The non-smokers could be passive smokers, but reported that they had never actively smoked.

4.2.1.2. Serum collection

Smoker and non-smoker donors were requested to provide 10 ml of blood after signing an informed-consent form. Blood samples were collected in plain serum separation tubes (BD Vacutainer™ Venous Blood Collection Tubes: SST™ Serum Separation Tubes: Hemogard); these tubes contain a special gel that separates blood cells from serum (Thermo Fisher Scientific). The tubes were centrifuged at 2000g for 10 minutes. Serum samples were aliquoted and stored immediately at -20°C.

4.2.1.3. Unstimulated saliva collection

Consenting donors (smokers and non-smokers) were requested to provide approximately 5 ml of saliva, which was collected over a period of 5-10 minutes. Donors ceased from eating and drinking for at least 1 hour prior to donation. After cleaning the mouth with mouthwash and rinsing with water over 5 min, donors provided saliva into plain separation tubes (Thermo Fisher Scientific) and the tubes kept on ice. Then, the saliva samples were centrifuged at 2600g for 15 minutes at 4°C. Samples were aliquoted and immediately frozen at -20 °C.

4.2.2. Assaying serum and saliva samples

Several serial dilutions of serum and saliva samples from healthy smokers and non-smokers were tested to determine the optimal dilutions for measuring each Ig class. The arbitrary fluorescence of each saliva and serum sample was compared to the standard curve of the serial dilutions for the specific Igs, as developed in the previous chapter.

To measure serum Ig levels, sera were diluted 1:100, 1:1,000, 1:20,000, 1:40,000, 1:100,000, and 1:500,000 in reagent diluent. Saliva was diluted to 1:1, 1:2, 1:10, and 1:1000 in the same reagent diluent.

4.2.3. Applying serum and saliva samples

Following a typical procedure for generating standard curves for each Ig tested in microarray format, the arbitrary fluorescence readings for serum and saliva samples

were interpolated with the standard curves, and their concentrations were calculated using Graphpad Prism 6.

Table 4.1: Demographics of the smoker subjects (a).

The information in this table was self-reported.

Number	Gender	Age	Number of cigarettes per day	Full inhalation of smoke/ Filter	Tobacco product	Years smoked
1	Male	32	10-12	yes/yes	Yes	15
2	Male	27	21	yes/yes	Yes	7
3	Male	33	10-20	yes/yes	Yes	15
4	Male	25	15	yes/yes	Yes	7
5	Male	34	5-10	yes/ no	Yes	10
6	Male	28	20	yes/ no	Yes	8
7	Male	47	< 10	yes/yes	yes	20
8	Male	34	40	yes/ no	Yes	18
9	Male	26	20	yes/ no	Yes	4
10	Male	28	15	yes/ no	Yes	10
11	Male	31	35-40	yes/ no	Yes	10
12	Male	30	30	yes/ no	Yes	16
13	Male	56	20	yes/ no	Yes	25
14	Male	28	30	yes/ no	Yes	13
15	Male	20	20	yes/ no	Yes	5
16	Male	24	20	yes/ no	Yes	7
17	Male	25	20-30	yes/ no	Yes	9
18	Male	22	1	yes/ no	Yes	2
19	Male	24	6-7	yes/ no	Yes	4
20	Male	21	1	yes/ no	Yes	5
21	Male	25	40	yes/ no	Yes	8
22	Male	28	20	yes/ no	Yes	8
23	Male	33	12	no/no	Yes	12
24	Male	36	20-40	yes/ no	Yes	10

Table 4. 2: Demographics of the smoker subjects (b).

The information in this table was self-reported.

Number	Gender	Age	Number of cigarette per day	Full inhalation of smoke/ Filter	Tobacco product	Years smoked
25	Male	33	20	yes/yes	Yes	33
26	Male	26	12-20	yes/no	Yes	10
27	Male	23	20	yes/no	Yes	6
28	Male	28	20	yes/no	Yes	10
29	Male	23	20	yes/no	Yes	5
30	Male	38	20	yes/no	Yes	20
31	Male	33	20	no/no	Yes	15
32	Male	37	20	yes/no	Yes	14
33	Male	30	4	yes/no	Yes	6
34	Male	29	30	yes/no	Yes	6
35	Male	20	30	yes/no	Yes	6
36	Male	30	24	yes/no	Yes	12
37	Male	23	25	yes/no	Yes	8
38	Male	46	20	yes/no	Yes	30
39	Male	30	20	yes/no	Yes	14
40	Male	29	15	yes/no	Yes	5
41	Male	44	20	yes/no	Yes	8
42	Male	26	20	yes/no	Yes	12
43	Male	35	20	yes/no	Yes	15
44	Male	28	20	yes/no	Yes	7
45	Male	36	20	yes/no	Yes	15
46	Male	45	20-25	yes/ no	Yes	12
47	Male	30	5-20	yes/ no	Yes	16
48	Male	36	10	yes/ no	Yes	10

4.3. Results

The overall distribution of serum Ig levels in the population was investigated using D'Agostino–Pearson omnibus normality test dependent on a Gaussian distribution, showed a non-normal distribution of IgA, IgG, IgM, and IgD.

4.3.1. Serum and saliva dilution-series results

Figure 4.1 shows a serum dilution of 1:40,000 was found to be optimal for measuring IgG and IgA, while a 1:200 dilution was optimal for IgM and IgD. In addition, a 1:2 dilution was suitable for detecting salivary Igs.

4.3.2. Serum sample results

The results (Figure 4.2) obtained for the serum samples from smokers and non-smokers, were compared using a Mann–Whitney test (2-tailed). Significant differences in the Ig levels were observed between the 2 groups in terms of IgG and IgD levels, which were lower in smokers than in non-smoker subjects ($P < 0.0001$, $P < 0.05$, respectively). However, IgM and IgA levels were significantly higher in smokers than in non-smoker subjects ($P < 0.0001$, $P < 0.05$, respectively).

4.3.3. Saliva samples results

The results (Figure 4.3) obtained for the saliva samples from smokers' and non-smokers' were compared using a Mann-Whitney test (2-tailed). Significant differences were observed in the Ig levels between the 2 groups, IgG ($P < 0.05$) and IgM ($P < 0.0001$) levels were lower in smokers than in non-smokers. IgA levels were higher in smokers than in non-smokers ($P < 0.05$) levels, and no significant differences were observed in their IgD levels.

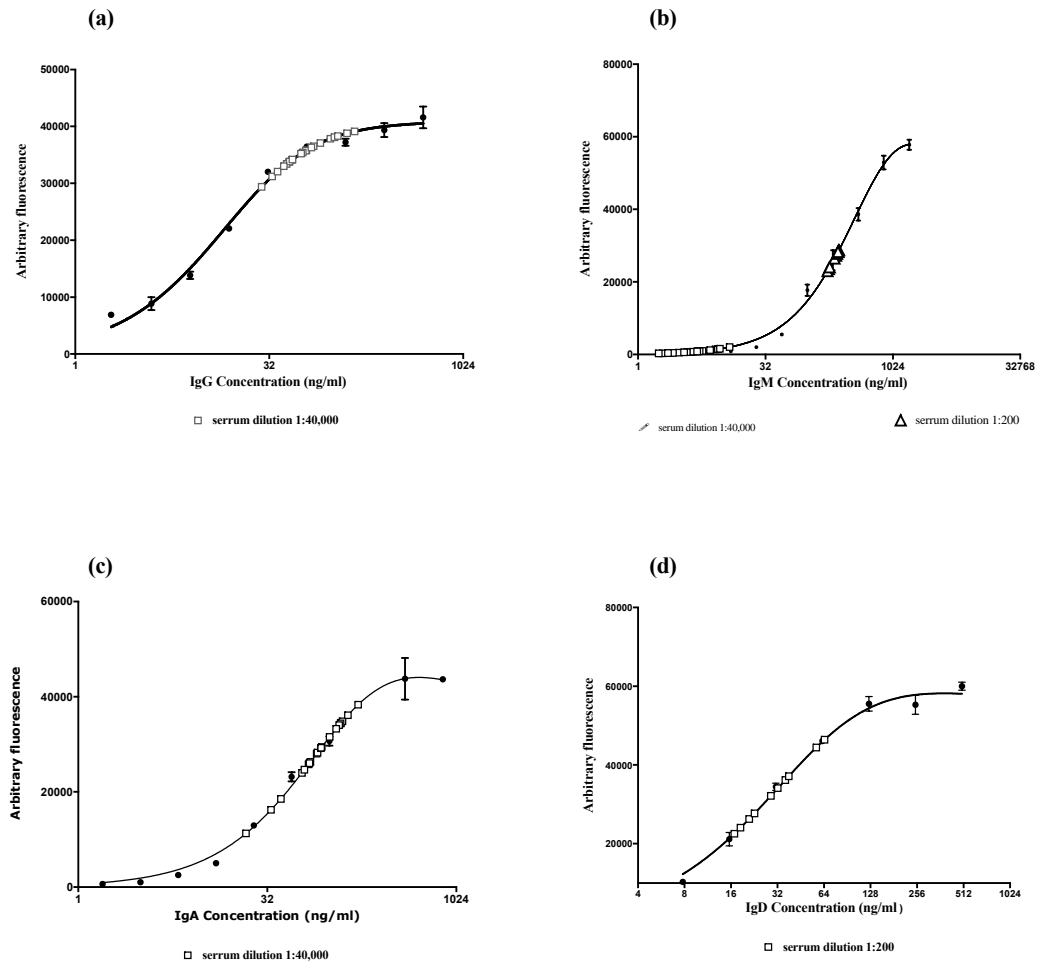


Figure 4.1: Determination of a suitable serum dilution.

The interpolated samples with a dilution of 1:40,000 fit in the middle of the curves for IgG and IgA, and a 1:200 dilution was used to measure IgM and IgD.

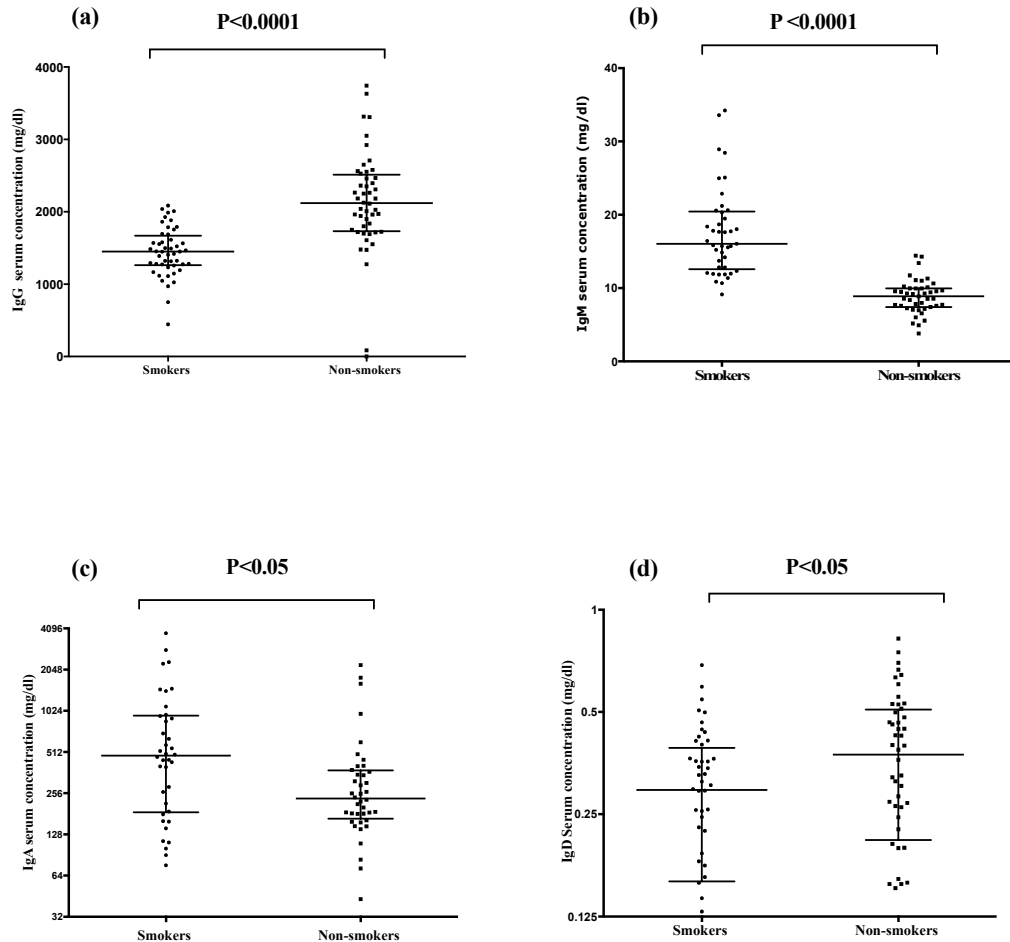


Figure 4.2: Comparing the Ig concentrations in serum samples from smokers and non-smoker subjects using a Mann–Whitney test revealed significant differences.

(a) Serum IgG concentrations were lower in smokers than in non-smoker subjects ($p < 0.0001$). (b) IgM concentrations were higher in smokers than in non-smoker subjects ($P < 0.0001$). (c) IgA concentrations were higher in smokers than in non-smoker subjects ($P < 0.05$). (d) IgD concentrations were lower in smokers than in non-smoker subjects ($P < 0.05$).

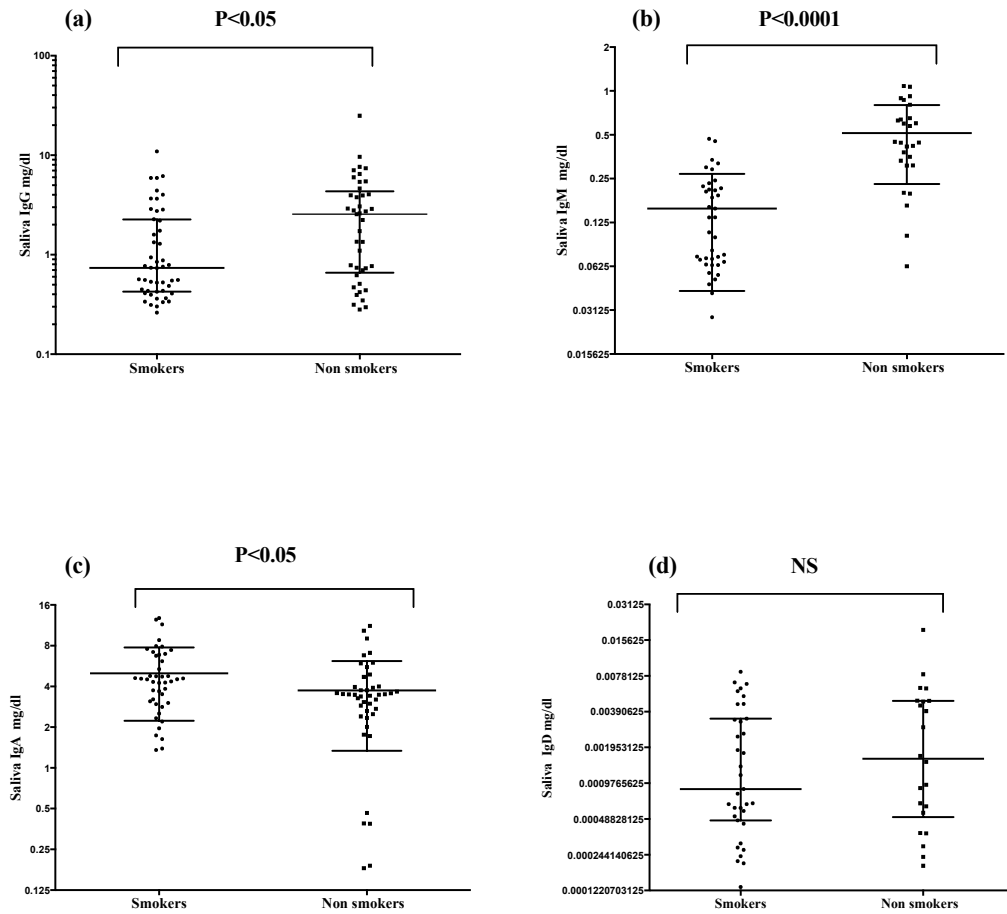


Figure 4.3: Comparing Ig concentrations in saliva samples from smokers and non-smoker subjects using a Mann–Whitney test revealed significant differences.

(a) IgG concentrations were lower in smokers than in non-smokers ($P < 0.05$). (b) IgM concentrations were lower in smokers than in non-smokers ($P < 0.0001$). (c) IgA concentrations were higher in smokers than in non-smokers ($P < 0.05$). (d) IgD concentrations were not significantly different between smokers and non-smokers.

4.3.4. Studying correlations between serum immunoglobulins

Figure 4.4 and figure 4.5 show the assessment of correlations between serum Ig levels for smokers and non-smokers, respectively. The Spearman coefficient correlation (2-tailed) was calculated for every 2 Ig isotypes in the serum samples. Only IgG and IgA from smokers showed a significant positive correlation ($P < 0.05$).

4.3.5. Studying correlations between saliva immunoglobulins

The correlations between saliva Ig levels were investigated using the Spearman coefficient correlation (2-tailed), which was calculated for every combination of 2 Ig isotypes in the saliva samples for smokers (Figure 4.6), and non-smokers subjects (Figure 4.7). The results (Figure 4.6) show that only IgG and IgA from smokers' saliva showed a significant positive correlation ($P < 0.05$).

4.3.6. Studying correlations between serum and saliva immunoglobulins

The Spearman coefficient correlation (2-tailed) was calculated for each of the Ig isotypes in the saliva samples vs serum samples from the same individuals. There were no significant correlations between Ig isotypes for the smokers' (Figure 4.8) or non-smokers (Figure 4.9) in these samples.

4.3.7. Correlation between immunoglobulin levels and smoking pack-years

To study the relation between serum Ig levels and smoking pack-years, the Spearman correlation coefficients (2-tailed) between each Ig class and smoking pack-years were calculated (Figure 4.10), and the Spearman correlation coefficients (2-tailed) for each saliva Ig isotype and smoking pack-years were calculated (Figure 4.11). These tests showed that the serum and saliva Ig levels were not significantly correlated with the smoking pack-years.

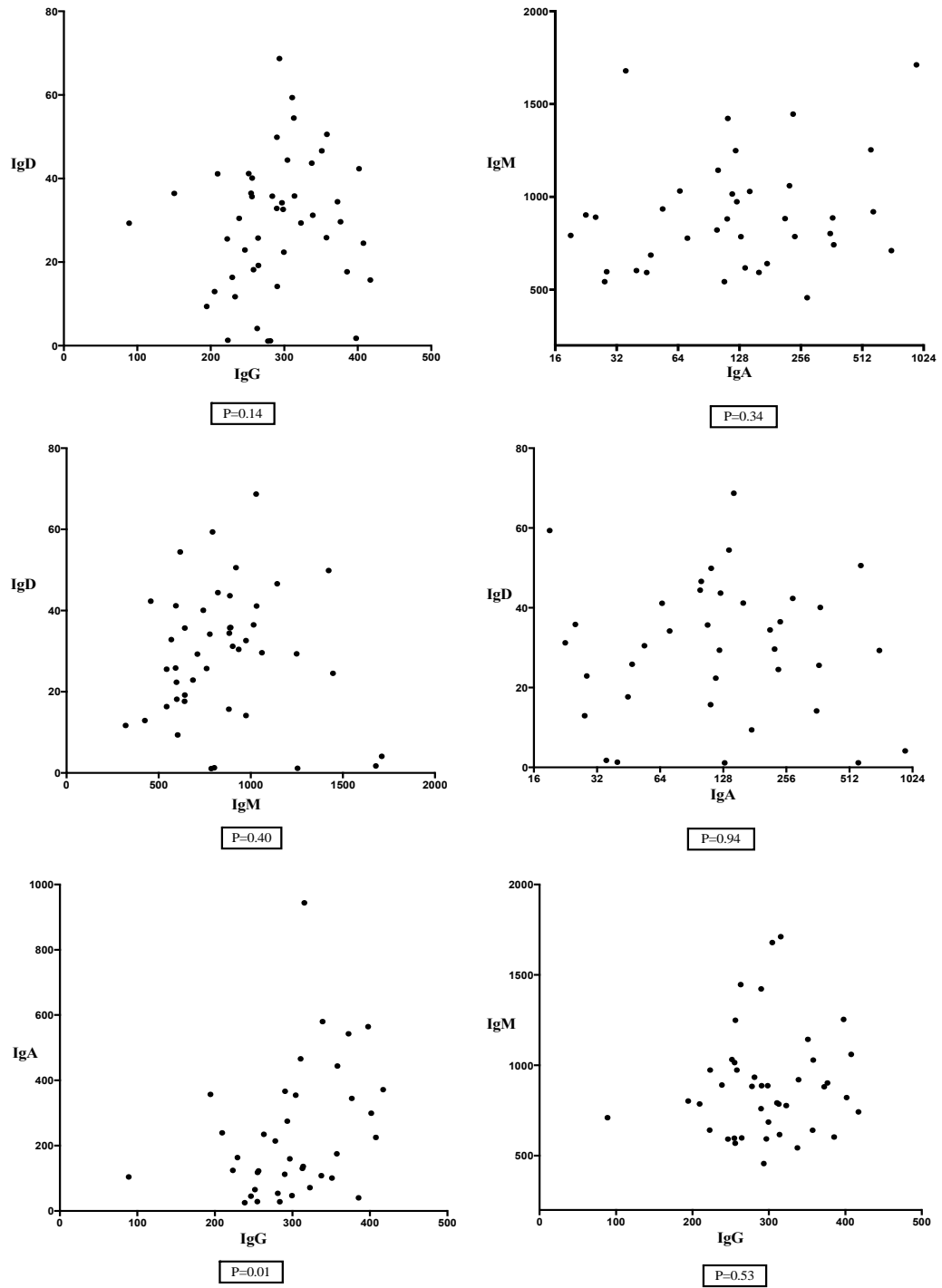


Figure 4.4: The Spearman correlation coefficient (2-tailed) between paired Ig isotypes for smoker's serum samples.

The results showed that the only significant positive correlation occurred between IgG and IgA ($P < 0.05$).

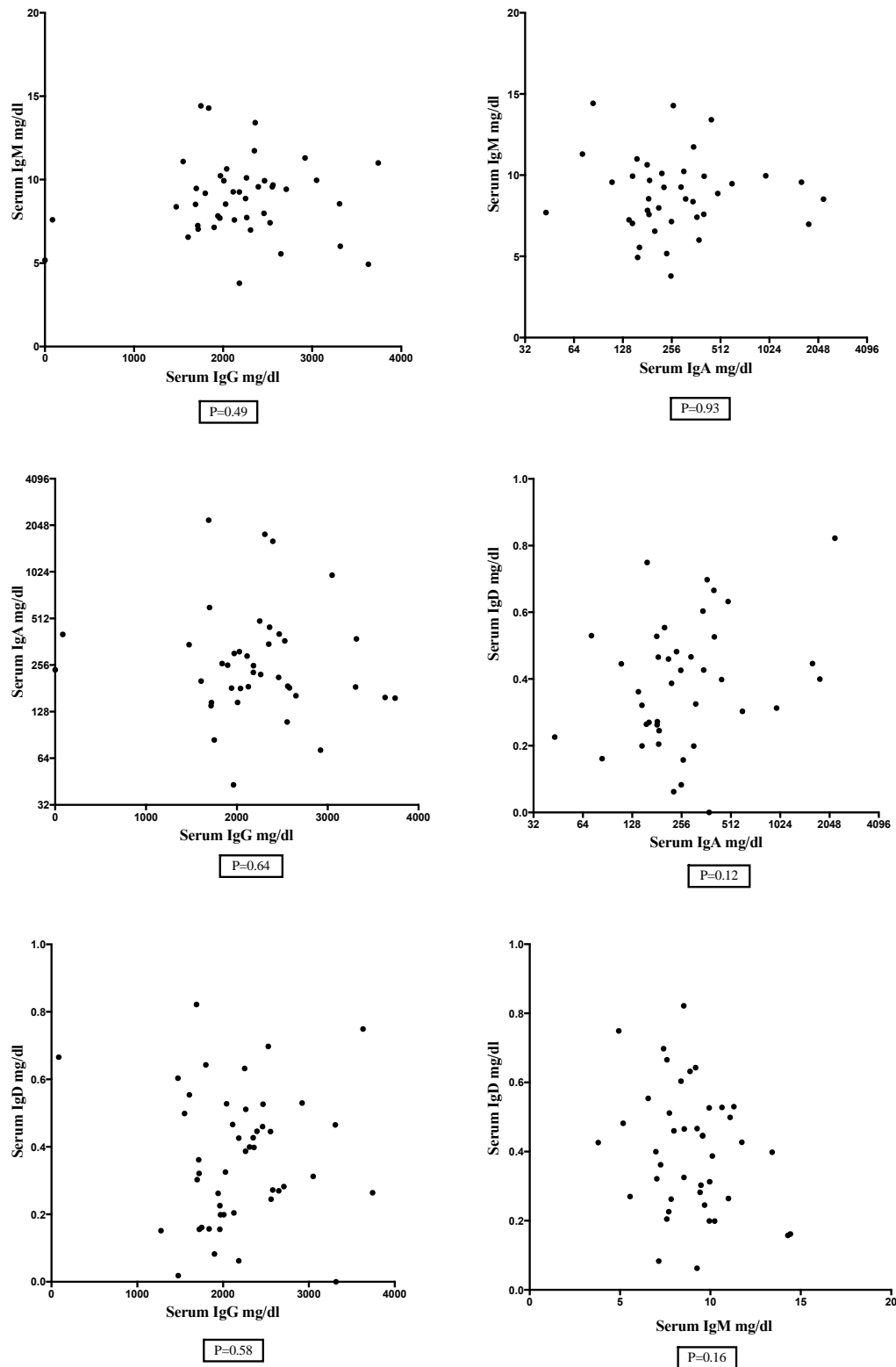


Figure 4.5: The plot of the Spearman correlation coefficient (2-tailed) between paired Ig isotypes for non- smoker's serum samples.

The results showed there are no significant correlations between immunoglobulin concentrations in the samples.

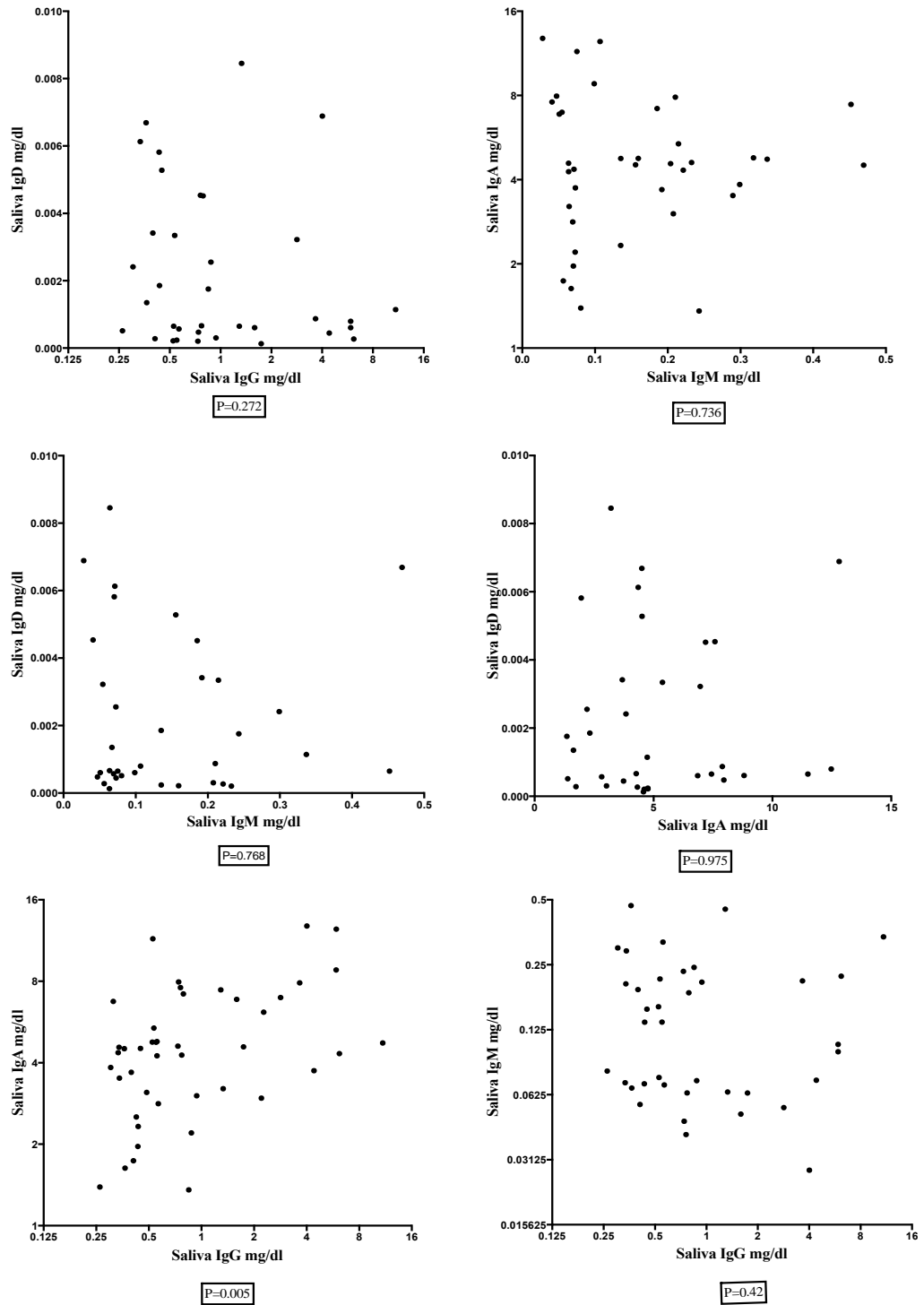


Figure 4.6: The plot of the Spearman correlation coefficient (2-tailed) between paired Ig isotypes for smokers' saliva samples.

The results showed that the only significant positive correlation occurred between IgG and IgA ($P < 0.05$).

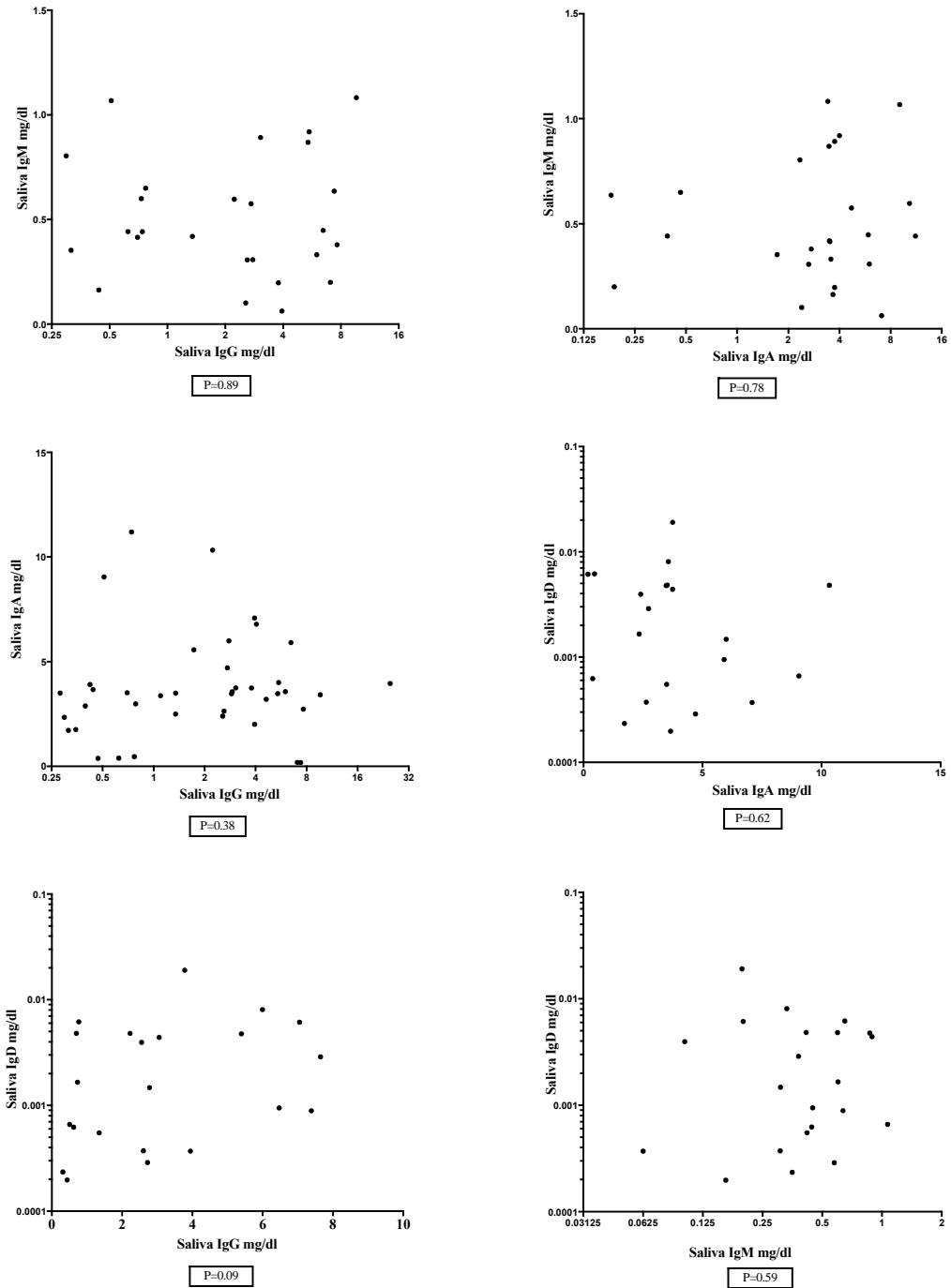


Figure 4.7: The plot of the Spearman correlation coefficient (2-tailed) between paired Ig isotypes for non-smokers saliva samples.

The results showed there are no significant correlations between immunoglobulin concentrations in the samples.

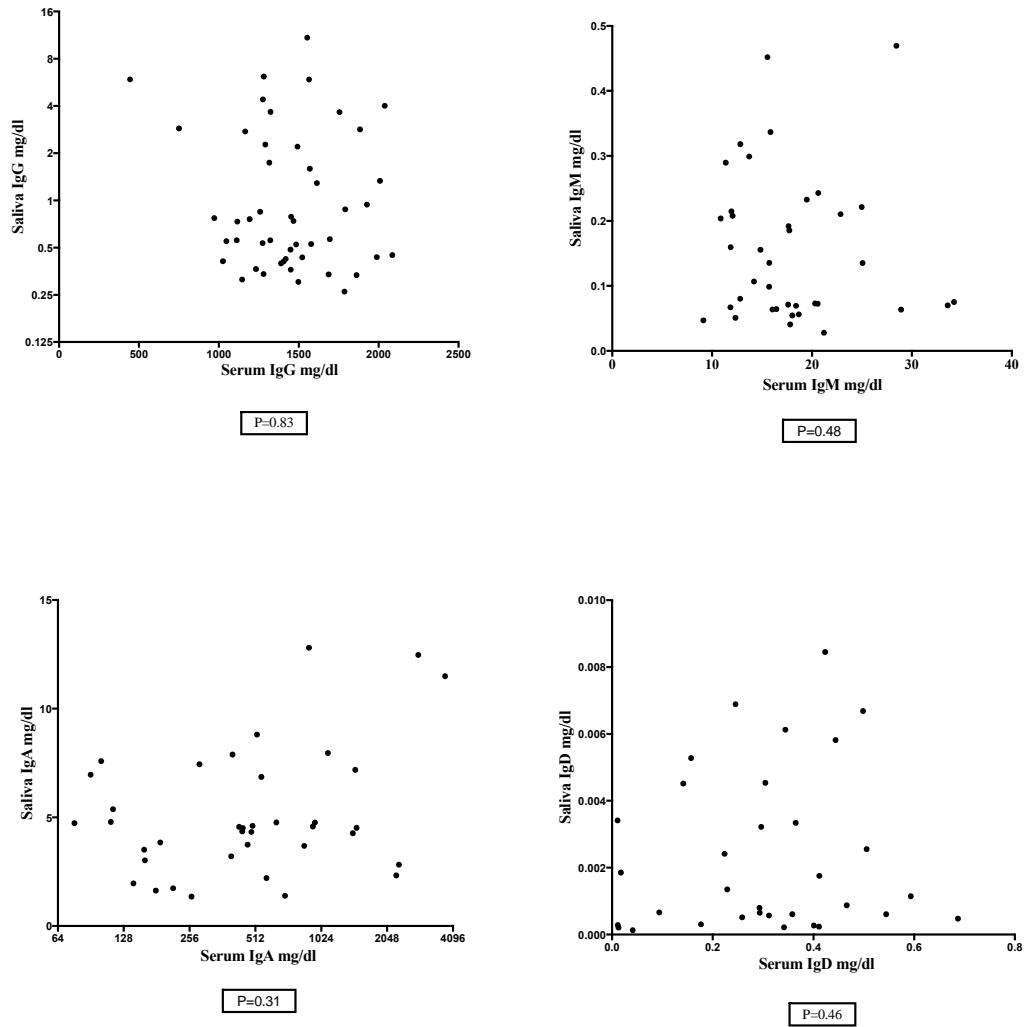


Figure 4.8: The plot of the Spearman correlation coefficient (2-tailed) between Ig isotypes in saliva and serum samples for smoker's subjects.

The results showed there are no significant correlations between immunoglobulin concentrations in the samples.

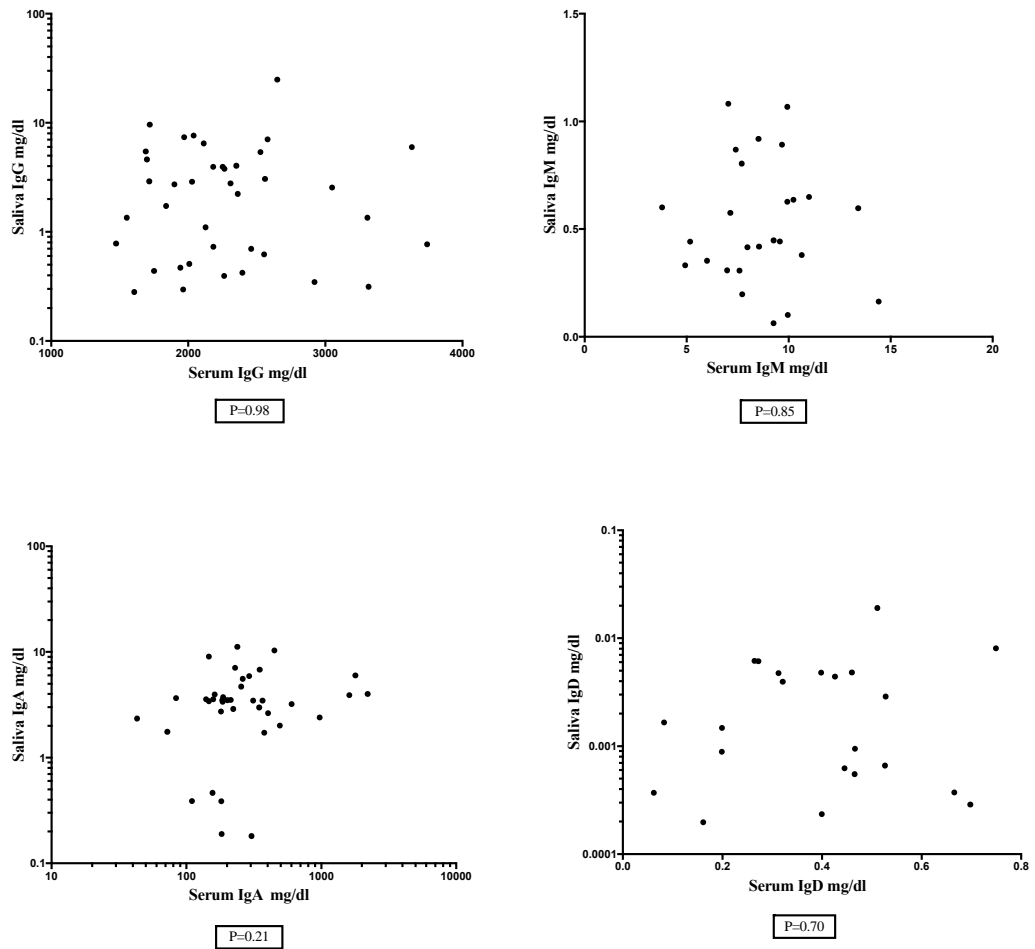


Figure 4.9: The plot of the Spearman correlation coefficient (2-tailed) between Ig isotypes in saliva and serum samples for non-smoker's subjects.

The results showed that the serum Ig levels were not significantly correlated with the saliva Ig levels.

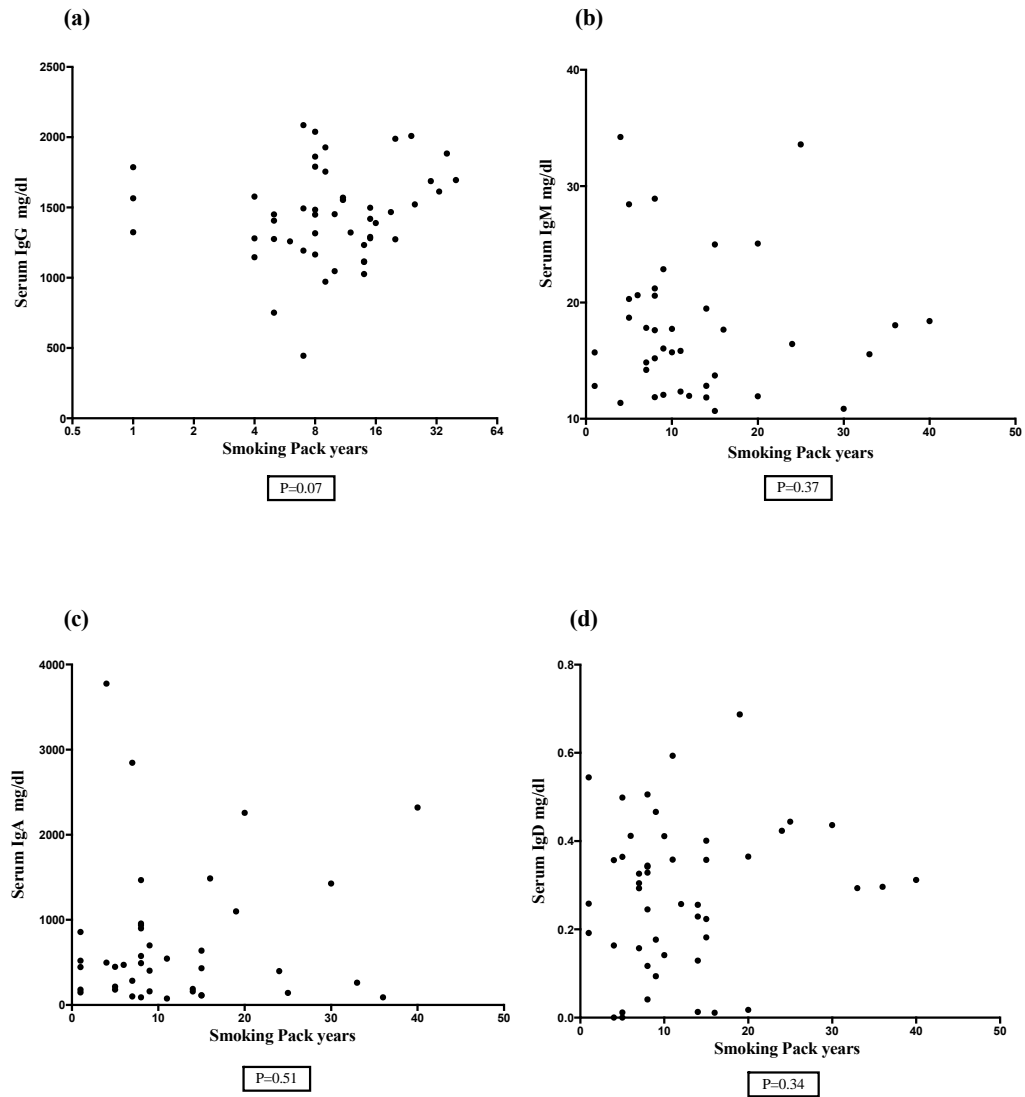


Figure 4.10: Studying the relation between serum Ig levels and smoking pack-years.

The Spearman correlation coefficients (2-tailed) for each Ig and smoking pack-years were calculated. The results showed that serum Ig levels were not significantly correlated with the smoking pack-years.

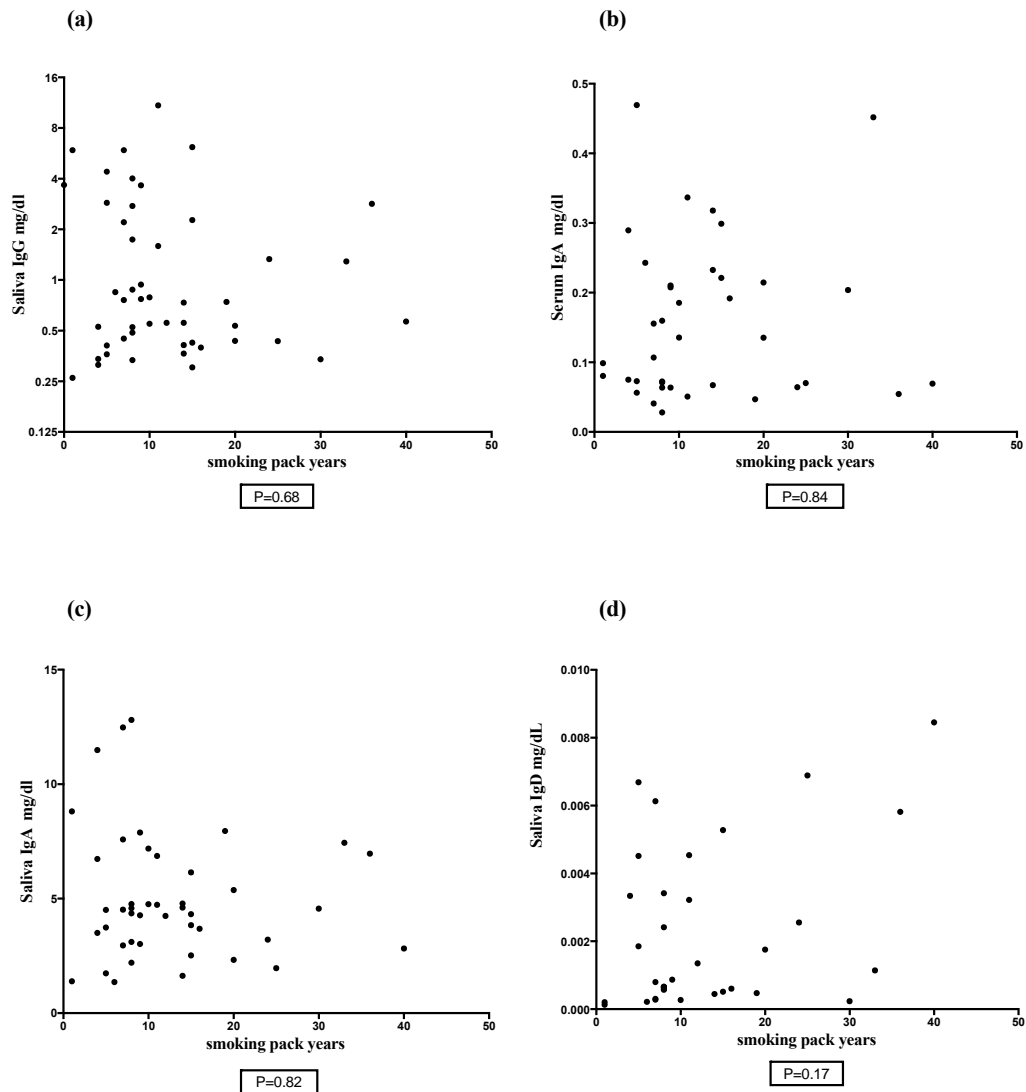


Figure 4.11: Studying the relation between saliva Ig levels and smoking pack-years.

The Spearman correlation coefficients (2-tailed) for each Ig and smoking pack-years were calculated. The results showed that the serum Ig levels were not significantly correlated with the number of pack years of smoking.

4.4. Discussion

Tobacco smoke contains numerous harmful components, such as oxidants and pro-oxidant compounds, which are efficient in generating free radicals and increasing oxidative stress [187, 188]. In fact, over 4000 different chemicals are released in tobacco smoke. These components include carbon monoxide, hydrogen cyanide, phenols, ammonia, formaldehyde, benzene (a) pyrene, nitrosoamines, nicotine, tar, heavy metals, radioactive products, poisons, and at least 48 known cancer-generating compounds [1, 189]. These harmful components not only affect active smokers, but also affect passive and involuntary smokers who inhale the smoke particulates from the environment [190]. It can be thought that long-term involuntary smoking may manifest with similar medical problems in active smokers [187]. Cigarette smoking is well known for its adverse health influences, and is considered a major risk factor for developing cancers, as well as cardiovascular and respiratory diseases. The dangerous effects of cigarette smoke include an increased risk for atherosclerosis [191, 192], coronary heart disease, and various neoplastic disorders, including lung cancer [192, 193]. There is evidence to indicate that maternal smoking during pregnancy leads to detrimental alterations in the placenta and fetus accompanied with low birth weight and increased risk of abortion and neonatal mortality in one-third in women who smoke during pregnancy compared with non-smoking women. Thus, increased risk of intrauterine growth retardation, prematurity, stillbirth and sudden infant death syndrome have been well recognized [194, 195]. It is also important to recognise that deleterious effects of smoking on infants after birth may be associated with the inhalation of cigarette smoke generated by other household members in addition to the mother. Studies have reported that cigarette smoking is one of the key mutagenic factors that can lead to damage to human genetic material. Thus, an improved understanding of the mechanisms whereby cigarette smoke negatively impacts human health is paramount, given its addictive nature and chronic cigarette-smoking consequences [196, 197]. Previous studies have revealed some effects that can explain the dangerous effects of tobacco smoke, such as its oxidative damage, the increased oxidation of LDLc, and elevated levels of fibrinogen, C-reactive protein (CRP), and uric acid [191, 198]. In addition, active cigarette smoking is associated with prognostic biochemical features of cardiovascular diseases, such as CRP elevation,

raised lipid peroxidation, inflammation, and variations in anti-oxidative enzyme activities [199, 200]. Therefore, the early detection of altered biochemical components in the body can be used as predictive biomarkers for smoking-related diseases. Even though the blood is considered a major source of biomarkers for diagnosing diseases, the saliva can also reflect the mucosal and systematic expression of analytes and give insights into disease diagnosis. Salivary Igs are derived from 2 sources: the salivary glands and the serum. Secretory IgA (sIgA) is produced by local plasma cells in the salivary glands and serves vital roles in the immune defence mechanisms that are active in the saliva. The minor salivary glands serve a crucial function in sIgA-mediated immunity of the oral cavity, and the cells in the parotid and submandibular glands are responsible for most the IgA found in saliva. 77 % of monomeric IgA in saliva is derived from the serum. In contrast, IgM and IgG in the saliva mainly originate from the serum, and some IgG might be locally produced in the gingiva and salivary glands. IgM and IgG exist in a lower abundance in saliva than does IgA [201]. The levels of salivary antibodies can reflect previous viral infections and can be used to diagnose viral infection, congenital infections, and reactivation of infection [202].

For my project, we hypothesized that smoking contributes to diseases through its effects on immune responses, inflammation, and Ig levels. Therefore, the purpose of this study was to compare Ig class levels in serum and saliva samples from smokers and non-smokers, as alterations in their production levels may yield valuable insights into the underlying mechanisms of smoking-related diseases, and also provide additional information useful for clarifying the basis of the toxic effects of cigarette smoking. To our knowledge, this is the first study to use a high-specificity microarray platform to compare the levels of Ig classes in serum and saliva samples from smokers and non-smokers. The microarray was optimised and validated using several tests, which facilitated the accurate and specific measurement of Ig levels.

Although the effects of cigarette smoking on the humoral system have been investigated previously, variations are apparent in the results obtained in different studies, and more research should be conducted on this subject. In terms of IgG, Charu Shrestha *et al.* (2014) showed that the long-term effects of smoking could lead to significant decreases in serum IgG levels. Smokers were found to have lower

serum IgG levels than observed in healthy control subjects and non-smokers with chronic periodontitis. In addition, it was shown that cigarette smoking could cause the suppression of B cell functions and the subsequent production of Igs. Therefore, the authors suggested that alterations in the levels of antibodies can explain the aggravations found in periodontal disease [203]. IgG levels were also lower in smokers with rheumatoid arthritis [204]. Another study also found that healthy smokers have lower serum IgG levels than do non-smoking individuals; however, no differences were found in the levels of serum IgA and IgM [133]. A previous study that focused on comparing the effects of smoking and alcohol consumption on serum Ig concentrations reported that alcohol consumption correlated with a significant increase in serum IgA levels, while the serum IgG and IgM levels decreased. However, the decrease in serum IgG levels, and to lesser extent IgM levels, was observed only in smokers. Further, smoking caused a decreased concentration of all Ig isotypes, and in a group who did not consume alcohol, smoking significantly correlated to decreased IgG concentrations [205]. Previous data showed that serum Ig concentrations tended to be lower in nicotine-consuming subjects, with IgG4 being significantly decreased in this group [206].

Arinola *et al.* (2013) reported that active cigarette smokers had elevated levels of serum IgG and IgM, compared with those observed in never-smokers. The authors explain that IgG and IgM promote activation of the complement system, which is essential for phagocytosis, microbe lysis, and toxin neutralization [207]. Considering that cigarette smoke contains many toxic components, the noted increase in IgG and IgM levels likely reflects an important mechanism in neutralising toxic tobacco components through complement-activation [187]. Other findings showed that smoking can increase the risk for oral infection with human papilloma virus and correlated with elevated levels of salivary IgG and lysozyme [208]. In a separate study, it was found that in a group of people who quit smoking, the quantity of lymphocytes decreased and NK activity against cultured melanoma cells increased. In addition, their serum IgG and IgM concentrations were significantly increased. Although there was no effect on the serum IgA concentration, the salivary IgA and IgG concentrations increased after smoking cessation. The NK activity and Ig concentrations showed no significant differences in the smoker group relative to that observed in the non-smoking group. Therefore,

these results demonstrated an inverse correlation between smoking status and immune function. These results may offer insights into the relationship of smoking status with an increased prevalence of certain malignant diseases and respiratory infection [209]. With respect to IgM levels, previous findings have indicated that no significant differences occur in serum IgM levels between smokers and non-smokers [138]. In addition, some research has indicated that significant increases occur in salivary IgM levels in smokers, compared with that observed in non-smokers, which may have an enhanced effect when combined with the low levels of salivary IgA [210]. The results from many studies have indicated that, in stimulated saliva (i.e. induced by chewing paraffin wax), individuals who have IgA immunodeficiency have increased IgM levels [129]. In terms of IgA, Barton *et al.* (1990) reported that in healthy individuals from Cairo, salivary IgA levels were lower in smokers compared to non-smokers, whereas salivary IgM levels were higher in smokers; salivary IgG levels were the same between smoking and non-smoking individuals; and no significant differences occurred between their levels in saliva. In addition, a strong negative correlation was observed between IgA levels and the number of cigarettes smoked per day. However, no relationship was identified between the salivary IgG or IgM levels and the number of cigarettes smoked per day. In addition, the effects of smoking cessation on Ig levels were studied, and it was found that differences in IgA levels were reversed after smoking cessation. It was also found that the salivary IgA levels in ex-smokers (for 2 years), who had been smoking 20 cigarettes per day, were higher than in current-smoker subjects. Moreover, 5 years later, the levels of salivary IgA were equivalent to those in non-smoker subjects. However, at 2 years post-smoking cessation, the levels of salivary IgM did not differ significantly from those of non-smokers. Nonetheless, at 5 years post-cessation, the IgM levels were significantly higher than in non-smokers [126]. Similar research conducted by a group in Edinburgh indicated that healthy smokers and patients with head and neck tumours, the majority of which were smokers, had lower salivary IgA and higher salivary IgM concentrations compared with those found in healthy non-smoking subjects. These data provide evidence regarding the effect of smoking on mucosal immunity. Based on the simple concept that IgM has very different properties compared to IgA, the increase in IgM levels could be interpreted as a compensatory action, and its elevated levels

could reflect immunopathology rather than naive compensation. We have not yet addressed the mechanism whereby cigarette smoking alters the Ig profile in saliva. Smoking, however, could impair the immune regulation of T cells on B cell differentiation and maturation. Alternatively, a study of the effects of cigarette smoking on cervical epithelial immunity showed that the variations can occur through gut antigen presenting cells in the afferent limb of immunity, as recently reported for uterine cervical epithelium. These authors assumed that an indirect mechanism was involved because the cervix and parotid gland are distant from the nearest sites directly affected by cigarette smoke. In addition, the effects of smoking show variation depending on whether an individual is a light, moderate, or heavy smoker [126, 211, 212]. Moreover, data from a previous study showed that tobacco smoking does not affect the compensatory rise in salivary IgG responses to an IgA deficiency in smoking and non-smoking individuals. According to a previous examination of salivary IgA in mixed unstimulated saliva, tobacco smokers showed decreased levels of salivary IgA when compared with that observed in the normal group [126, 127]. Conflicting evidence, however, has shown that no significant differences occur in salivary IgA and IgG, while salivary IgM was significantly lower in a smoking group compared to that in a non-smoking group [131]. Lie *et al.* (2002) reported similar levels of salivary IgA between smokers and non-smokers participating in a gingivitis trial [132]. Giuca *et al.* (2014) compared salivary Ig levels between smokers and non-smokers versus their periodontal disease status, and they reported that smokers have lower saliva IgA, IgG, and IgM levels, as well as worsened periodontal conditions than non-smokers. Although there is little evidence that salivary Igs exert a protective effect against periodontitis and that not all saliva components are produced by the salivary glands, it can be concluded that the worsened periodontal health conditions of these patients can be attributed in part to decreased protection resulting from a reduction in the quantity of salivary Igs [213].

The levels of serum IgG and IgM were reported to increase significantly after smoking cessation, but serum IgA levels remained similar. However, salivary IgG and IgA levels rose after smoking cessation [209]. Contradictory evidence indicates that no significant difference occurred in serum IgA levels between smoking and non-smoking individuals [134]. Indeed, reduced IgA levels were detected in

bronchoalveolar lavage fluid (BALF) in smoking individuals [63]. Conversely, other evidence indicated that enhanced levels of IgA were present in the BALF [136, 214]. In addition, the salivary IgA levels showed a gradual reduction from controls to smokers, which may be used as a diagnostic marker in smokers and patients with recurrent aphthous ulcers [128]. Richmond *et al.* (2014) reported that patients with COPD who still smoked lacked sIgA, which could lead to enhanced COPD pathogenesis and likely rendered the epithelium more vulnerable to damage [215]. Results from a study performed to evaluate the levels of salivary IgA in tobacco chewers, tobacco smokers, and a control group showed that in unstimulated whole saliva, the IgA levels decreased more in tobacco users than in control group, and tobacco smokers had much lower salivary IgA levels compared to tobacco chewers [216]. A study conducted in 2015 found that in the lungs, cigarette smoke may delay quick mucosal IgA responses and enhance the severity of influenza viral infection via inhibition of BAFF, a cytokine essential for B cell production and maturation [217]. Prior research has also shown that cigarette smoking is an essential factor in the development of IgA nephropathy, where IgA accumulates and damages the kidneys, and smoking cessation should be considered for patient treatment and recovery [218]. Several studies have provided evidence that serum IgA levels are lower in smokers than in non-smokers by 10–20 % [123, 124]. Moreover, serum IgA levels are higher in healthy smoking individuals, compared with patients with COPD that also smoke [130].

With regards to IgD, previous research has shown that, in cigarette smokers, the levels of serum IgD are twice as high as in non-smokers. However, their levels return to normal after smoking ceases. Other studies have provided evidence that, in COPD patients, the levels of IgD in serum and nasal secretions are higher than those in control individuals. These findings suggest that reduced IgD levels may be related to a slowed progression of COPD. In contrast, salivary IgD levels do not show significant differences between smokers and non-smokers [141].

The results shown in this chapter demonstrated that significant differences occur between Ig levels in smokers and non-smokers. In serum samples, the IgG and IgD levels of smokers were lower than those of non-smoker subjects, while IgM and IgA levels were higher in smoker subjects. In addition, the results obtained with saliva samples showed the same patterns, except for the IgM levels, which were

lower in smokers than in non-smokers. The increased serum IgM in smokers could help to neutralize cigarette smoke toxins, as suggested by Arenolate 2013. We can suggest that cigarette smoke may inhibit class switching to IgG production, therefore there would be more IgM in the smokers' serum samples and less IgG. Alternatively, CS may preferentially promote class switching to IgA rather than IgG, consistent with inflammatory effects of CS on the respiratory mucosa and our observation of raised IgA levels in both the serum and saliva of smokers. CS might also promote increased activation of naïve B cells, which would help to account for the increased serum levels of IgM in smokers. Conversely, the levels of IgM were lower in the saliva of smokers than the non-smokers, which might be a compensatory effect for the increased levels of salivary IgA. IgM diffuses into secretions at a much lower rate than IgA, and is also more susceptible to enzymatic cleavage. Thus, it may be that cigarette smoke has an immediate effect on the upper part of the respiratory tract, which is exposed directly to CS and that leads to changed salivary antibody levels, and consequently this can cause respiratory inflammation and disease. Collectively, our data indicate that cigarette smoking suppressed B cell secretion and impaired humoral immunity. Consequently, the IgG and IgD levels decreased, and therefore CS may have different effects in B cells, leading to different IgM levels in the saliva and serum.

As discussed above, different studies have reached different conclusions about the effect of smoking on Ig class levels. Our findings are consistent with those of some of these previous studies, but differ from others. Clearly, a range of variables may influence the observed effects of CS on Ig levels. These include the nature and conditions of the assays used. Advantages of our study, using protein microarrays, is the ability of this technique to be used to assay large numbers of samples simultaneously, thereby reducing inter-assay variation; also, the large dynamic range of signal detection means that the Ig levels can be accurately measured over a wide range of concentrations without loss of sensitivity at the highest and lowest concentrations. Furthermore, the ability to perform many sample replicates simultaneously and to exclude poor-performing replicates in the microarray based on defined and valid exclusion criteria (e.g. spot circularity, pixel density, etc.) improves the accuracy and reliability of this technique [219, 220].

4.5. Conclusions

The mechanism whereby smoking affects Ig concentrations remains incompletely understood. Prior research has provided evidence that Ig levels (particularly IgG levels) decreased with increased smoking, and these effects were reversible after smoking cessation [205, 206, 209, 221]. Currently, it remains unclear which constituents of cigarette smoke are responsible for these effects; however, nicotine has been implicated as a key factor in animal and *in vitro* studies [206, 222, 223]. Our findings demonstrated that cigarette smoking caused alterations in serum and saliva Ig levels; thus, smoking causes both systemic effects and effects in the oral mucosa. These findings shed insight into how smoking causes diseases through altering B cell secretions.

Overall, the IgM levels increased in the serum of smokers, which may help to neutralize the toxic components in cigarette smoke, but an altered immune response caused a decrease in both the IgG and IgD levels in the smokers. This could be because CS inhibits the mechanism of class switching. In addition, CS is a main cause for lung disorders, which could explain the increased IgA levels in the serum and saliva of smoker subjects. On other hand, our finding concluded that CS caused a decreased level of IgM in saliva, which could be related to the compensatory balance between IgM and IgA. However, whether Ig levels are affected *in vitro* in response to CSE or nicotine in a similar manner to that seen in smokers is not clear, as to date, no *in vitro* studies relating to this issue have been reported. Therefore, to investigate the mechanism of action of CSE on Ig secretions, and whether it has a similar impact on Ig secretions when CS is exposed directly to B-cells alone or indirectly in the presence of other immune cells such as T cells and antigen presenting cells, we decided to conduct a preliminary *in vitro* study with isolated B cells and peripheral blood mononuclear cells (PBMCs).

5. Studying the Effects of CSE/Nicotine on B-cell Secretions *in vitro*

5.1. Introduction

The constituents of cigarette smoke enter the human body through the pulmonary alveoli and then enter the bloodstream and become distributed to all tissues via capillary vessels. The particulates in smoke can affect cells within blood, such as T cells, B cells, and monocytes, which can then exert systemic effects on other organs. The results of previous studies have shown that smoke particulates cause up-regulation of many nicotine receptors on the surface of B cells [72, 224].

In addition, long-term exposure to nicotine can inhibit the proliferation and development of B cells, which then reduces antibody secretions [74]. As a result, smoking is the main agent promoting the development of some diseases, such as COPD and emphysema and the dysregulation of B cell functions may play a contributing role. Moreover, previous data have indicated that smoking can lead to carcinogenesis, as well as respiratory diseases in women (more than in men) [225-227]. In addition, smoking can impair the fertility of women and lead to early menopause [228, 229]. *In vitro*, PBMCs have been used to study the effects of cigarette smoke on immune cells and their gene expression [230-232]. *In vivo*, the influence of cigarette smoking in females on circulating B cells resulted in the inhibition of ICOSLG, TCF3, and VCAM1 gene functions [75].

Following the analysis of Ig concentrations, which revealed that variations in their levels are associated with cigarette smoking, we became interested in examining the *in vitro* effects of CSE on antibody secretion from stimulated B cells. B cells were stimulated in both T-cell-dependent and -independent manners.

5.2. Materials and methods

5.2.1. Materials

The materials used for the *in-vitro* experiments are summarized in Table 5.1.

Table 5.1: List of materials used for *in vitro* experiments.

Materials	Sources
Ethylene diamine tetra-acetic acid (EDTA)	Sigma-Aldrich
Foetal bovine serum (FBS)	Sigma-Aldrich
Histopaque-1077	Sigma-Aldrich
L-glutamine	Sigma-Aldrich
70 % ethanol	Sigma-Aldrich
Trypan blue	Sigma-Aldrich
Negative B cell isolation kit	Miltenyi Biotec GmbH (Bergisch Glad- bach, Germany) (Cat. no. 130-091-151)
MACS buffer	50 ml phosphate- buffered saline (PBS), 200ul ethylenediaminetetra-acetic acid (EDTA) and 830ul of 30 % □ BSA
BSA	Sigma-Aldrich
Biotin-Antibody Cocktail (Cocktail of conjugated monoclonal antibodies against CD2, CD14, CD16, CD36, CD43, and CD235)	Miltenyi Biotec GmbH (Bergisch Gladbach, Germany)
Hanks' Balanced Salt Solution	Sigma- Aldrich
LS separation column	Miltenyi Biotec
RPML-1640 medium	Sigma-Aldrich
HEPES	Sigma-Aldrich
Penicillin	Sigma-Aldrich

5.2.2. Methods

5.2.2.1. Isolation of PBMCs

Buffy coat were obtained from anonymous blood donations via the Regional Blood Transfusion Service (Sheffield), who provided ethical approval, within 24h of collection. PBMCs were isolated from 50 ml buffy coat samples diluted into 150 ml PBS and mixed well. Then, the blood samples were transferred slowly using a syringe attached to a filling tube into 6 sterile 50-ml centrifuge tubes each

containing 10–15 ml Histopaque (Histopaque-1077; Sigma-Aldrich, Irvine, UK). The blood samples were then centrifuged at $800 \times g$ for 30 min. Subsequently, the white blood cell layer was collected with a sterile Pasteur pipette and placed into new sterile tubes, and 25 ml Hank's Balanced Salt Solution was used to wash the cells (Hanks 123K2368; Sigma-Aldrich, Irvine, UK). The samples were centrifuged again at $800 \times g$ for 10 min, and the supernatant was discarded. Then, the samples were washed twice in PBS with centrifugation at $300 \times g$ for 10 min, after which they were counted.

5.2.2.2 Cell counting of viable cells

First, a haemocytometer slide was cleaned with 70 % ethanol. Then, 10 μ l of cells and 10 μ l of Trypan blue were mixed and pipetted onto the haemocytometer slide and covered. Trypan blue was used to selectively colour dead cells blue. The slide was placed under a microscope, and the mean numbers of cells in three large squares containing 4 smaller squares each were counted. The concentration of cells in the original sample was calculated as follows:

$$\text{Number of cells/ml} = \text{number of cells counted} \times \text{dilution factor} \times 10^4$$

5.2.2.3. Magnetic separation of B cells

To separate B cells, isolated PBMCs were subjected to immunomagnetic negative selection of B-lymphocytes, using the Negative B Cell Isolation Kit from Miltenyi Biotec GmbH (Bergisch Gladbach, Germany) (Catalogue No. 130-091-151). Briefly, the cell pellet from the previous step was resuspended in cold MACS buffer (50 ml PBS, 200 μ l EDTA, and 830 μ l of 30 % μ l BSA; Sigma-Aldrich, Irvine, UK) at a ratio of 40 μ l / 1×10^7 cells. Then, Biotin-Antibody Cocktail (cocktail of biotin-conjugated monoclonal antibodies against CD2, CD14, CD16, CD36, CD43, and CD235a [glycophorin A]) was added at a ratio of 10 μ l / 1×10^7 total cells. Next, the samples were incubated for 5 min in a refrigerator (2–8 °C). Subsequently, 30 μ l MACS buffer and 20 μ l Anti-Biotin Micro Beads were added / 1×10^7 total cells, and the suspension was incubated for an additional 10 min in the refrigerator (2–8 °C). Then, the cell suspension was resuspended in 1 ml MACS buffer and loaded onto a cold LS separation column (Catalogue No. 120-000-472; Miltenyi Biotec). Magnetic cell sorting was initiated after setting the magnet on the holder and the column on the cold magnet. A 3 ml LS column was equilibrated with MACS buffer,

and the cells were then passed through the column. The column was rinsed 3 times with 3 ml LS column MACS buffer to elute the B cells. After the third wash, the column was removed from the magnet and placed in a new sterile tube, and 3 ml MACS buffer/LS column was added and plunged through to remove the remaining leukocytes. The B cell samples were then centrifuged at $300 \times g$ for 5 min to remove the MACS buffer. Finally, the B cells were resuspended at a concentration of $1 \times 10^6/\text{ml}$ in RPMI 1640 complete medium (Catalogue No. RNBB7148; Sigma-Aldrich, Irvine, UK) supplemented with 10 mM HEPES, 100 IU/ml penicillin, 100 $\mu\text{g}/\text{ml}$ streptomycin, 2 mM L-glutamine (Sigma-Aldrich, Irvine, UK), and 10 % foetal bovine serum (FBS). Normally, the B cell preparation was 95 % pure ($\text{CD}19^+$), with less than 0.5 % $\text{CD}3^+$ (T cells) and $\text{CD}14^+$ (monocytes).

5.2.2.4. Treatment of cell cultures with CSE or nicotine

Unfractionated PBMCs and purified B cells were suspended in complete RPMI 1640 medium and cultured in 24-well microtitre culture plates (Costar, High Wycombe, UK). Two ml of cell suspension was added to each well (2×10^6 cells/well). Cells were stimulated with IL-4 (100 U/ml), anti-CD40 mAb (1 mg/ml), and with or without pokeweed mitogen. Some wells were treated with 1 % or 3 % concentrations of CSE. Nicotine (N3876, Sigma-Aldrich, Irvine, UK) was added at final concentrations ranging from 12–200 $\mu\text{g}/\text{ml}$ to assess the effects of this important constituent of cigarettes on B cells, and some wells were incubated with an equal amount of complete medium as a negative control. Cultures were routinely maintained at 37 °C in a 5 % CO_2 humidified atmosphere for up to 8 days. Then, the supernatants were harvested by centrifugation at $400 \times g$ for 10 min at 4 °C. Cell-free supernatants was stored at -20 °C until aliquots were used for IgG, IgM, IgA, and IgD determinations.

5.2.2.5. Preparation of CSE

A special technique was used to extract the components of cigarettes. To accomplish this, Marlboro Red cigarettes were prepared first by cutting off the filters, and the cigarettes were placed into one side of an in-house prepared smoking apparatus. The second side of the apparatus was attached to a 50 ml syringe, and the third side was attached to a tube containing 10 ml phenol red-free RPMI medium (Sigma-Aldrich, Irvine, UK). The extraction was done by drawing

cigarette smoke into the syringe and then bubbling it into the medium until the entire cigarette was consumed (Figure 5.1). Then, the medium was mixed with the smoke and filtered with a 0.45 μm filter. Subsequently, from this mixture, 5 serial dilutions were prepared in 5 Eppendorf tubes, and their absorbance were measured using a NanoDrop ND-1000 spectrophotometer. Then, a standard curve was generated, and the tube with an absorbance of 0.15 was chosen and considered as 100 %. Finally, the CSE with a concentration of 1 % or 3 % was used to treat the cells (Figure 5.2).

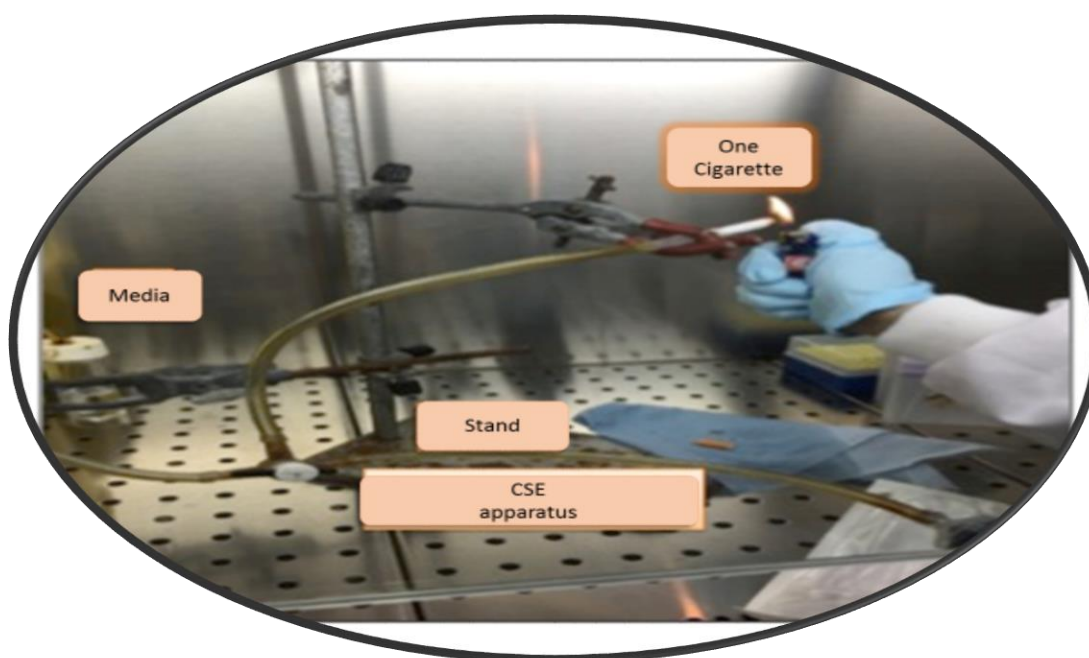


Figure 5.1: Preparation of CSE.

A specialised smoking apparatus was set up: the cigarette was located on one side, the second side was connected to a 50 ml syringe, and the third side was contained a tube containing 10 ml phenol red-free RPMI medium (Sigma-Aldrich, Irvine, UK). The flow between the three connections was controlled by a tap, so the cigarette smoke could be into the syringe, and then the direction of the tap changed to allow the smoke to be bubbled into the medium. The process was repeated until the entire cigarette was consumed.

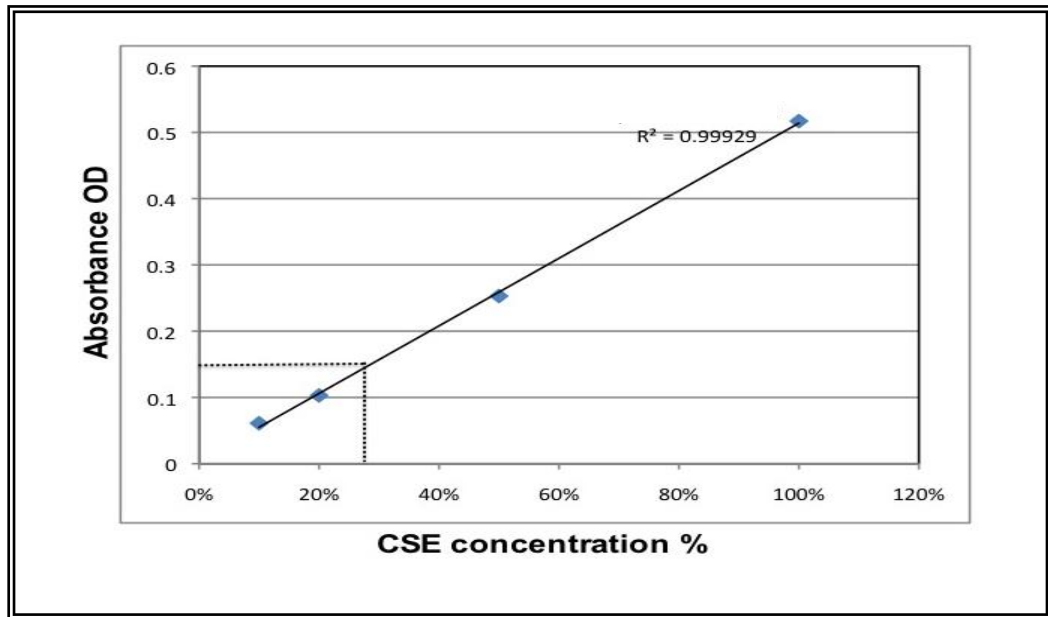


Figure 5.2: Using a NanoDrop spectrophotometer to analyse CSE absorbance in a standard curve.

The standard curve was generated after determining the absorbance of known concentrations (serial dilutions) of CSE that were extracted in the lab. One Marlboro Red cigarette was used each time for the extraction, during which the smoke was bubbled into phenol red-free medium. Serial dilutions of the CSE were prepared. Then, a NanoDrop (ND1000) spectrophotometer was used to measure the absorbance values. From the standard curve, it was possible to estimate the concentration equal to an absorbance of 0.15, which usually ranged from a 20–25 % concentration of the total extract.

5.2.2.6. Determination of Ig levels in cell-free supernatants

Cell-free supernatants from PBMC and B cell cultures were used to measure the levels of IgG, IgM, IgA, and IgD. The ELISA technique discussed above (chapter 2) was used to generate standard curves, and the samples were analysed after a 1:10 dilution in sample diluent.

5.2.2.7. Statistical analysis

Statistical analysis was performed using Prism 6 software. The median values of the three groups (treated samples with 3 % CSE, treated samples with nicotine, and untreated samples) was compared to that of control-group samples that were treated only with stimulators, using the analysis of variance test.

5.3. Results

As shown in Figures 5.3 and 5.4, when supernatants were harvested from PBMC and B-cell cultures, the samples treated with 3 % CSE or nicotine showed lower IgG, IgM, and IgA concentrations, compared with the group treated only with stimulators (control group), IgD was not detected in these samples. By using Dunnett's multiple comparisons test for samples collected from PBMCs the results showed that IgG levels (Figure 5.3) were significantly lower in samples treated with CSE ($P < 0.05$) or nicotine ($P < 0.05$), and in the non-treated samples ($P < 0.05$), compared with the stimulated control group. Also, IgM levels (Figure 5.2) were significantly lower in samples treated with CSE ($P < 0.0001$), or nicotine ($P < 0.0001$), and in the non-treated samples ($P < 0.0001$), compared with the stimulated control group. IgA appeared to be lower in the treated samples, but there were no significant differences between the groups.

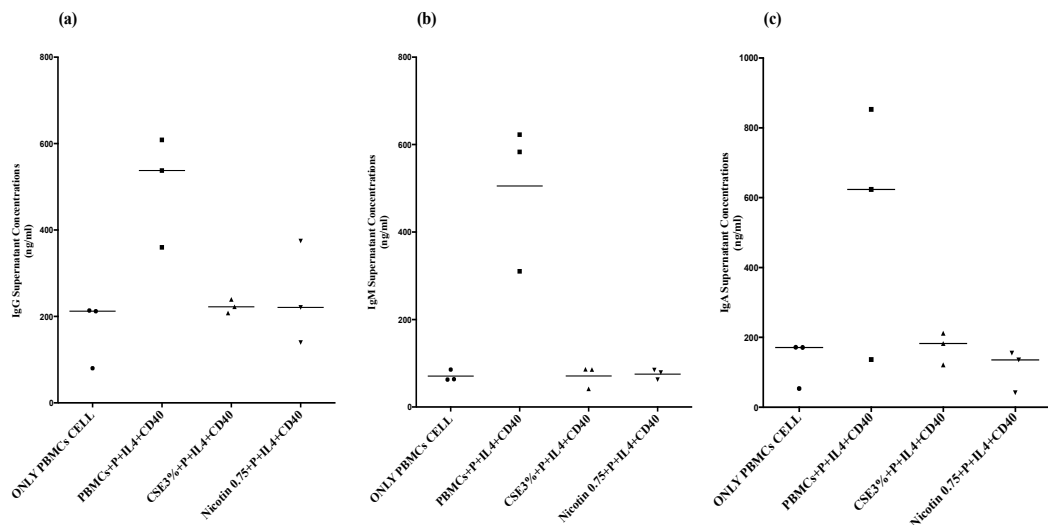


Figure 5.3: Stimulated human PBMCs were incubated for 8 days in the presence of anti-CD40, IL-4, PWM, 3 % CSE, or nicotine.

IgG, IgM, and IgA were detected in the supernatants by performing an ELISA. a) IgG levels were lower in samples treated with CSE or nicotine, compared with samples treated only with stimulators. b) IgM levels were lower in samples treated with CSE or nicotine, compared with samples treated only with stimulators. c) IgA levels were lower in samples treated with CSE or nicotine, compared with samples treated only with stimulators.

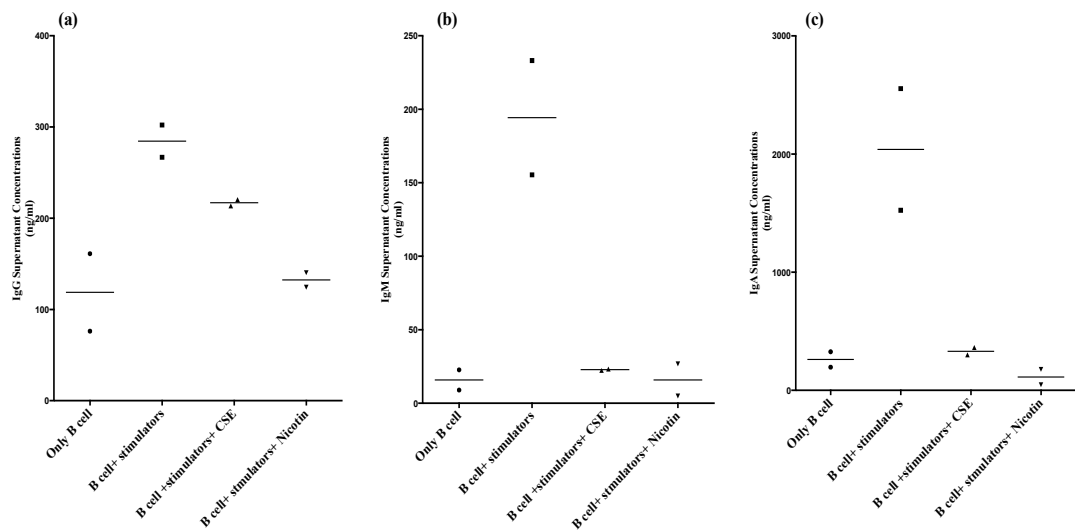


Figure 5.4: Stimulated human B cells were incubated for 8 days in the presence of anti-CD40, IL-4, PWM, 3 % CSE, or nicotine.

IgG, IgM, and IgA were detected in the supernatants by performing an ELISA. a) IgG levels were lower in samples treated with CSE or nicotine, compared with samples treated only with stimulators. b) IgM levels were lower in samples treated with CSE or nicotine, compared with samples treated only with stimulators. c) IgA levels were lower in samples treated with CSE or nicotine, compared with samples treated only with stimulators.

5.4. Discussion

To our knowledge, this is one of the first studies to concentrate on the effects of CSE/nicotine on the production of Ig from human cells *in vitro*. In this study, we showed that CSE and nicotine, as one of the most important toxic components in CSE, suppressed the production of IgM, IgG, and IgA in both PBMCs and purified B cells, which were treated with IL-4, pokeweed mitogen, and anti-CD40 as stimulators. Our results were in agreement with those of another report of suppressed Ig production by some toxic components when mouse B cells were treated with the aryl hydrocarbon receptor (AhR) agonist 2-(1'-H-indole-3'-carbonyl)-thiazole-4-carboxylic acid methyl ester in the presence of IL-4 and anti-CD40 [233]. The molecular mechanism of how AhR suppress B cell differentiation into secreting plasma cells is not fully understood. It has been reported that B cell suppression could occur via transcriptional regulators of the gene network that controls the differentiation of B cells into plasma cells [233]. Moreover, it was also reported that immune cells express nicotinic receptors; macrophage nicotinic receptors can control the production of pro-inflammatory cytokines, and in T lymphocytes, a deficiency of cell activation was correlated with nicotine activity. Shock *et al.* (2003) found that nicotinic receptors in the form of nicotinic acetylcholine receptors were expressed in B lymphocyte-derived cell lines, and the existence of these receptors was implicated in both the pre-immune state and the activation of B lymphocytes, possibly through mediating signalling through the co-stimulatory molecule CD40, which could contribute to immune depression in cigarette smokers [72-74].

5.5. Conclusions

Our results showed that CS had direct effects on the production of immunoglobulins, and it led to inhibition of the secretion of IgG, IgM and IgA from B-cells. Unfortunately, it was only possible to perform a limited number of experiments on the effects of CS and nicotine on antibody production *in vitro* in the context of this study, as shown in figures 5.2 and 5.3; it is therefore clear that further experiments are needed in the future to confirm the results presented here.

6. General Discussion

The aim of this study was first to investigate the effects of cigarette smoke on Ig class expression in the oral cavity and systemically through measuring the concentrations of different Ig classes in saliva and serum specimens in healthy smoking and non-smoking subjects, using a highly sensitive antibody microarray technique that was developed in this study, and subsequently to study the underlying mechanism of action *in vitro* on B cell Ig secretion. We applied our optimized and validated antibody microarray platform to the sera and saliva from healthy smokers and non-smoker subjects. The results of this study revealed the significant differences between Ig classes between smokers and non-smokers in serum and saliva samples. Thus, these alterations in Ig levels could contribute to inflammation and disease in smokers. In fact, previous studies have shown that smokers have up to 10-20 % lower serum IgA, IgG and IgM levels than non-smokers [1]. However, another study showed that there was higher IgM levels in the serum of smokers than non-smokers [126]. It has also been shown that salivary IgA levels decrease 7 days after stopping smoking, but they return to normal levels two weeks after smoking cessation [1, 234]. In addition, Bouvet *et al.* (2002) reported that sIgA, which is a crucial Ig class produced in human secretions at luminal sites involved in oral, gastrointestinal, and respiratory secretions, is prompted after antigen (Ag) translocation from the lumen to mucosa-associated lymphoid tissue. However, IgG in secretions is derived from the serum or mucosa, and increased IgG classes at the mucosal surface could be a defensive response. These findings have indicated that tobacco smoke affects both systemic and mucosal immunity with fluctuations occurring in mucosal and systemic antibody production, which enhances the probability of progression of respiratory disease [1, 219].

In the current study, serum and saliva IgG levels were lower in smokers than in non-smoker subjects. These findings are consistent with the suppressive effects of the toxic components of CS on B cells leading to impaired humoral immune responses. We observed increased IgM levels in the serum samples of smokers, compared with those measured in non-smoker subjects. This result may reflect the crucial role of IgM in neutralizing the toxic components in cigarette smoke.

Furthermore, the increase in systemic/circulating IgM levels at the same time as a decrease in systemic IgG levels may indicate that components of cigarette smoke that enter the blood stream and infiltrate the systemic secondary lymphoid tissues (e.g. lymph nodes and spleen) either suppress B cell class switching from IgM to IgG production and/or are selectively toxic to memory B cells compared to naïve B cells. By contrast, IgM levels were lower in saliva samples from smokers than non-smoker subjects, which could have been due to the compensatory mechanisms between IgM and IgA since the results showed higher IgA levels in the saliva (as well as the serum) of smokers. These results are consistent with cigarette smoke being a significant cause of inflammation in the respiratory mucosa that is manifested through increasing IgA levels, resulting from exaggerated mucosal immune responses in smokers and more class switching to IgA. This offers an alternative explanation, to that given above, for the effects of CS on Ig class switching: rather than inhibiting all class switching in IgM⁺ B cells, the effect of CS components may be to polarise switching away from IgG and towards IgA. One explanation for this could be the effect of CS on T cells and the cytokines they produce in the context of the regulation of Ig class switching. The Th1 cytokine IFN- γ promotes IgG production, whereas the Th2/Treg cytokines IL-5 and TGF- β promote IgA production. It would therefore be interesting to investigate the effects of CS on these T cell subsets. Indeed, our group has preliminary evidence that shows raised median levels of TGF- β relative to IFN- γ IL-4 and IL-10 in smokers compared to non-smokers and ex-smokers (Figure 6.1) [235]. In terms of TGF- β there are different studies that have reported an inducing effect of CS on the production of TGF- β Li *et al.* (2000), found that, in an animal model, after 3 months of smoke exposure TGF- β levels were increased significantly compared to the normal control, which means this could be considered as one of the mechanisms for smoking-induced chronic bronchitis and emphysema [236]. Similar evidence comes from Mortaz *et al.* (2011), which showed that CS stimulates the production TGF- β *in vitro* [237]. Moreover, a study in the Chinese population (2013) emphasized that there was an important role of the TGF- β signalling pathway in colorectal cancer, and that was induced with smoking [238]. In terms of IFN- γ Ouyang *et al.* (2000) indicated that there were inhibitory effects of CS on the production of IFN- γ and IL2 in human PBMC treated samples [239].

IgD levels in the serum samples were lower in smokers compared with non-smokers, and not significantly different between smokers and non-smokers in the saliva samples. Studies have shown that IgD has an important role in protecting the upper respiratory tract. It is known that human IgM-IgD⁺ B cells are uncommon in peripheral blood, but they make up 20–25 % of the human upper respiratory tract mucosal B cells. These cells are located in the tonsils, nasal cavities, lachrymal glands and salivary glands, however they are seldom identified in non-respiratory mucosal tract locations [240, 241]. Secreted IgD contributes to mucosal immunity and can bind to microbial virulence factors as well as pathogenic respiratory bacteria and viruses [58]. The number of IgD-producing B cells in respiratory mucosa is raised in patients suffering from IgA deficiency [241]. Also, secreted IgD activates unknown receptors on various innate immune cells, and previous studies have shown that IgD binds with myeloid cells and T cells [58]. Recent studies have shown that IgD binds to specific receptors on basophils, mast cells and, to a minor degree, to monocytes, neutrophils and myeloid dendritic cells [241, 242].

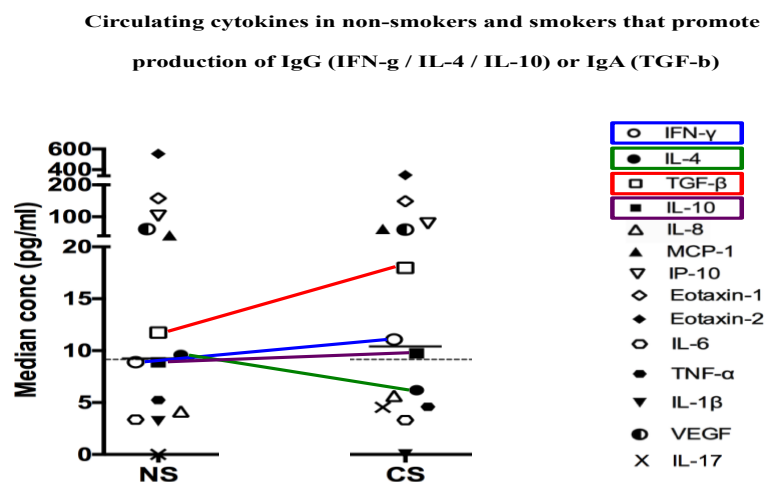


Figure 6.1: Graph of the levels of circulating cytokines in smokers and non-smokers.

The results showed that TGF- β levels in smokers were higher than in non-smokers, which may relate to the higher level of IgA in smoking subjects. This figure has been adapted from Selvarajah *et al.* [235].

Overall, our results support the concept that cigarette smoke has modulatory effects on both systemic and mucosal humoral immunity leading to alterations of B cell

secretions.

The effects of CS may be partly explained by the fact that it contains numerous oxidants that are capable of interacting with numerous biomolecules, including DNA, RNA, lipids, proteins, dietary antioxidants, and numerous endogenously synthesized biomolecules, such as glutathione [1, 243, 244]. Therefore, exposure to CS is considered to be a key source of cellular oxidative stress that results in lung inflammation [1, 245]. Oxidative stress can be described as an increased exposure to oxidants with a decreased anti-oxidant efficiency. Two main phases are recognized in CS: a tar phase and a gas phase; both phases are rich in oxygen-centred, carbon-centred, and nitrogen-centred free radicals and non-radical oxidants. These include various compounds that can cause increased generation of various reactive oxygen species (ROS) such as superoxide ($O^{\cdot-}$), hydrogen peroxide (H_2O_2), and hydroxyl (OH^{\cdot}) and peroxy (ROO^{\cdot}) radicals [1, 246]. These ROS can stimulate oxidative damage in the form of lipid peroxidation. Durak *et al.* (2002) showed that smoking generates a significant oxidant load in erythrocytes by increasing production of malondialdehyde, which is a marker of lipid peroxidation and oxidative stress. In turn the toxic free radicals and oxidants in CS cause detrimental reactions with unsaturated fatty acids and several other oxidation-sensitive molecules, moreover, CS contains copper and iron elements, which stimulate hydroxyl radical production [247]. The oxidative stresses carry out crucial functions in inflammatory responses to CS, and inflammation itself stimulates oxidative stress in the lungs, also gene polymorphisms can function as inflammatory mediators or antioxidant genes [1, 248]. In addition, other research found that oxidants can activate NF- κ B, which plays an essential role in regulating the expression of inflammatory genes stimulated by CS [1, 249]. Furthermore, oxidative stress can activate regulatory components such as nuclear factor (NF- κ B) DNA-binding activity, which can regulate the expression of several cytokines such as IL-6 and IL-8 [250]. NF- κ B can also induce activator protein-1 expression, which can control various inflammatory genes that are overexpressed in response to CS [1, 251]. Therefore, researchers conclude that there are a number of mechanisms working together to explain how diseases are associated with smoking.

To understand the mechanism of action of CS on B cells, and whether these effects

produce biological consequences or not, the final part of our study involved preliminary experiments on the effects of CSE, especially nicotine as a major toxic component of CS, on B cell secretions *in vitro*. The levels of IgG, IgM and IgA in the supernatants from PBMCs and purified B cells were analysed.

Interestingly, the results showed that reduced production of all the Ig classes occurred when the cells were treated directly with total CSE or nicotine alone compared with that observed in control cells. Therefore, our results indicated that CS affected the activation of B cells in either the presence or absence of other immune cells, which was shown by the levels of Ig classes decreasing in both PBMC and the purified B cell supernatants, with the latter indicating that the effect was not necessarily T cell-dependent. Thus, the experiments indicate that CS and nicotine can have direct suppressive effects on B cells, although effects on T cells and antigen presenting cells, which would also indirectly affect B cell functions, are not excluded. These findings agreed with those from other studies, where nicotine was considered a main immunosuppressive constituent of CS, as chronic nicotine treatment resulted in decreased antibody production [1, 186, 252]. Moreover, it has been demonstrated that alveolar macrophages in smokers show reduced function as antigen-presenting cells [253]. This outcome may ultimately result in impaired T cell function, and, consequently, decreased humoral immune responses.

Our preliminary finding that CS and nicotine suppress production of all the Ig classes examined (IgG, IgM and IgA) is different from our findings in smokers, particularly for IgA, which was elevated in both the serum and saliva of the smokers. This suggests that the effects of CS *in vivo* are complex and are not fully replicated by our *in vitro* model.

6.1. Conclusions and future work

In conclusion, this study involved the use of specific reagents for an antibody microarray and many experiments were performed to ensure the validity and reliability of our method, which resulted in the development of a new antibody microarray protocol, using a specific blocking buffer, and capture and detection antibodies. The results of this study demonstrated significant differences in Ig levels

between smokers and non-smoker subjects. The mechanism of the decreased IgG concentrations in smokers may be due, at least in part, to smoking reducing the activation of existing memory B cells, or lack of appropriate help from T cells. The increased levels of IgM in smokers' blood could be due to CS constituents blocking class switching or enhancing the activation of naïve B cells. Regarding the increased IgA production in smokers, IgA plays a crucial role in inflammation, especially in lung disease, and our results are in agreement with other studies that have shown increased class switching towards IgA in healthy smokers, whereas studies found that COPD smokers had increased switching towards IgG [130]. Brandsma *et al.* (2009) observed a negative correlation between B cells and Treg cells, and they reported Tregs can inhibit B cells, with increased class switching of memory B cells to IgA [254].

One of the possible mechanisms can be summarized as follows: CS is a highly-concentrated source of ROS which interacts with body biomolecules through numerous mechanisms, depending on the amount and way of smoking, and the susceptibility of smokers. These interactions might change the humoral immunity, and then by direct or indirect processes cause inflammation and increased incidence of multiple diseases.

Regarding the *in vitro* work that focused on the influence of CSE/nicotine on PBMC and B cells, a protocol was developed to determine the concentrations of cells, stimulators, and CSE. The results showed a drop in the secretion of Ig classes examined in cells exposed to CSE/nicotine, compared to that observed in the control groups. This may be explained by the fact B cells express nicotinic receptors that recognize and bind cigarette toxins and result in impaired B cell proliferation, and consequently causing decreased Ig production [72, 73].

Finally, the results of this study confirmed the impact of CS on both systemic and mucosal immunity, with alterations shown in mucosal and systemic antibody production, which promote inflammation and, consequently, the incidence of respiratory disorders. In addition, CS exposure can affect the proliferation of B-cells through direct or indirect pathways.

Certain limitations are associated with this study. For example, this study was performed with men only; further studies should include female populations. In addition, in this study, we used a questionnaire to assess the smoking status of the patients, which is a subjective measure. Other, more accurate assessments can be used in the future. A small number of samples were used for the *in vitro* experiments. The use of a larger sample size could engender greater confidence in the results. In conclusion, the current observations suggested that cigarette smoking is associated with altered B cell functions and, consequently, changes in Ig production.

The results of this thesis suggest several interesting avenues of investigation that could consolidate the results of this study in the future:

Antibody microarray: The reagents that were used in our developed antibody microarray were designed to investigate large numbers of samples in very small quantities because of the numerous experiments needed to test the accuracy of these components and ensure the precision of the results. Our protocol can be used in the future as an indicator of early changes in Ig levels, to study the incidence of multiple diseases, and potentially to monitor diseases in their earliest stages.

Stability of the printing on the slides: An important parameter that should be investigated is how long the capture antibody can remain stable on the poly-L-lysine slides, and how to maximise this stability. This would then facilitate the bulk production and storage of slides coated with the capture antibody for future use.

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Appendix

The ethical approval letter, which has been issued to collect serum and saliva samples.

KINGDOM OF SAUDI ARABIA Ministry of Higher Education KING ABDULAZIZ UNIVERSITY Faculty of Medicine	 	المملكة العربية السعودية وزارة التعليم العالي جامعة الملك عبد العزيز كلية الطب
Ref. :		الرقم :
Date : / /		التاريخ : ١٤٣٣ / / هـ
Encl. :		الرفقت :

UNIT OF BIOMEDICAL ETHICS
Research Committee

TO : Principal Investigator: Ms. Nesrin Tarbiah (Biochemistry)
Supervisor : Dr. Ian Todd Co-investigator : Ms. Lucy Fairclough
Date: Tuesday, August 20, 2013 CC : Vice-Dean, University / Hospital Director & File & Expedite approval File

From : Professor. Hasan Alzahrani
Co-investigator : Dr. Paddy Tighe

RE : "Investigation of the effects of cigarette smoke on Immunoglobulin levels in saliva and serum, in relation to COPD hospital ." (Reference No 1204-13)

The above titled research/study proposal has been examined with the following enclosures:

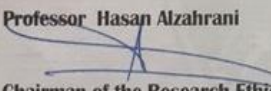
- The study protocol
- Consent form

The REC recommended granting permission of approval to conduct the project along the following terms:

1. Any amendments to the approved protocol or any element of the submitted documents should NOT be undertaken without prior re-submission to, and approval of the REC for prior approval.
2. Monitoring: the project may be subject to an audit or any other form of monitoring by the REC.
3. The PI is responsible for the storage and retention of original data of the study for a minimum period of five years.

The Organization & operating procedure of the KAU, Faculty of Medicine - Research Ethics Committee (REC) are based on the Good Clinical Practice (GCP) Guidelines.

PLEASE NOTE THAT THIS APPROVAL IS VALID FOR ONE YEAR COMMENCING FROM THE DATE OF THIS LETTER.

Professor Hasan Alzahrani

Chairman of the Research Ethics Committee

(HA-02-J-008) No of Registration At National Committee of Bio. & Med. Ethics.
Mohammed al searee (Reference No 1204-13)

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